Down-regulation of the expression of alcohol dehydrogenase 4 and CYP2E1 by the combination of α-endosulfan and dioxin in HepaRG human cells

Eléonore A. Attignon[a,b], Emilie Distel[a,b], Béatrice Le-Grand[a,b], Alix F. Leblanc[a,b,1], Robert Barouki[a,b,c], Eliandre de Oliveira[d], Martine Aggerbeck[a,b], Etienne B. Blanc[a,b,*]

[a] INSERM UMR 1124, Toxicologie Pharmacologie et Signalisation Cellulaire, 45 rue des Saints Pères, 75006 Paris, France

[b] Université Paris Descartes, Sorbonne Paris Cité, UFR des Sciences Fondamentales et Biomédicales, 45 rue des Saints Pères, 75006 Paris, France

[c] AP-HP, Hôpital Necker-Enfants Malades, Service de Biochimie Métabolique, 149, rue de Sèvres, 75743 Paris, France 75006 Paris, France

[d] Proteomics platform, Barcelona Science Park, Barcelona, Spain

[1] present address: Ohio State University, College of pharmacy

* corresponding author: etienne.blanc@parisdescartes.fr, telephone: (+33) 1 42 86 33 60, fax (+33) 1 42 86 38 68. Université Paris Descartes, INSERM UMR 1124, 45 rue des Saints Pères, 75006 Paris, France.

Abstract

Pesticides and other persistent organic pollutants are considered as risk factors for liver diseases. We treated the human hepatic cell line HepaRG with both 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) and the organochlorine pesticide, α-endosulfan, to evaluate their combined impact on the expression of hepatic genes involved in alcohol metabolism. We show that the combination of the two pollutants (25 nM TCDD and 10 µM α-endosulfan) led to marked decreases in the amounts of both the mRNA (up to 90%) and protein (up to 60%) of ADH4 and CYP2E1. Similar results were obtained following 24 hours or 8 days of treatment with lower concentrations of these pollutants. Experiments with siRNA and AHR agonists and antagonist demonstrated that the genomic AHR/ARNT pathway is necessary for the dioxin effect. The PXR, CAR and estrogen receptor alpha transcription factors were not modulators of the effects of α-endosulfan, as assessed by siRNA transfection. In another human hepatic cell line, HepG2, TCDD decreased the expression of ADH4 and CYP2E1 mRNAs whereas α-endosulfan had no effect on these genes. Our results demonstrate that exposure to a mixture of pollutants may deregulate hepatic metabolism.

Keywords

Pollutant mixture; ADH4; liver; persistent organic pollutant; organochlorine pesticide; TCDD

Abbreviations

ADH: Alcohol Dehydrogenase; AHR: Aryl Hydrocarbon Receptor; ALD: Alcohol Liver Disease; ARNT: AHR Nuclear Translocator; CAR: Constitutive Androstane Receptor; CYP: Cytochrome P450; ER: Estrogen Receptor; OCP: Organochlorine Pesticide; POP: Persistent Organic Pollutant; PXR: Pregnane X receptor; TCDD: 2,3,7,8 tetrachlorodibenzo-*p*-dioxin.

1. Introduction

Over the past several decades, exposure to environmental pollutants has become a chronic insult for humans. Persistent Organic Pollutants (POP) are resistant to biological and chemical degradation, are lipophilic and accumulate in the adipose tissue. Several of the POPs act as endocrine disruptors and, thus, may modify the normal physiology of the organism and could lead to long-term pathologies such as chronic liver diseases (Wahlang et al., 2013). Indeed, epidemiological studies suggest that POPs can contribute to the increase in incidence of several pathologies, among which are type 2 diabetes and obesity (Lee et al., 2014; Magliano et al., 2014).

To date, most studies have dealt with exposures to a single contaminant (or families of contaminants). The effects of complex mixtures have been investigated only recently in several *in vitro and in vivo* models (De Boever et al., 2013; Lyche et al., 2013) even though animal models imperfectly reflect the human species.

Members of the dioxin family of POPs can be by-products of industrial processes such as organochlorine pesticide (OCP) manufacturing (Schecter et al., 2006). 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent member of the dioxin family and α-endosulfan is the most toxic endosulfan isomer (Sutherland et al., 2004). These two POPs are known to act *via* different xenosensors. TCDD is a classical ligand of the aryl hydrocarbon receptor (AHR) (Barouki et al., 2007) whereas α-endosulfan has been described as acting through the pregnane X receptor (PXR) and/or the estrogen receptor (ER) and, to a lesser extent through the constitutive androstane receptor (CAR) (Casabar et al., 2006; Coumoul et al., 2002; Lemaire et al., 2006; Savary et al., 2014). In humans, the liver is the primary target organ due to its detoxification function (Baillie and Rettie, 2011) as well as a storage site of

POPs, together with the adipose tissue (Agency of Toxic Substances and Disease Registry, 1998, Addendum 2012). A transcriptomic study using the combination of TCDD and α-endosulfan, showed a drastic down-regulation of the expression of several genes, including alcohol dehydrogenases and CYP2E1, in human hepatic HepaRG cells (Ambolet-Camoit et al., 2015). The molecular mechanisms involved in those inhibitions are still not known. Work from our laboratory in the same HepaRG cell line, a recognized model for the human hepatocyte for the study of both drug metabolism (Anthérieu et al., 2010, Guillouzo 2007) and intermediary metabolism (Rogue et al., 2012; Samanez et al., 2012), showed that treatment with TCDD decreases the levels of both mRNA and protein of several alcohol dehydrogenases *via* AHR (Attignon et al., 2017).

CYP2E1 (EC 1.14.13.n7) and ADH (EC 1.1.1.1) are phase I xenobiotic metabolizing enzymes which catalyze the oxidation of xenobiotic (alcohols and drugs) and endobiotic molecules (retinoids and prostaglandins for CYP2E1 ; neurotransmitters, ω-hydroxy fatty acids and hydroxysteroids for ADH) (Cheung et al., 2005; Dey and Kumar, 2011; Höög and Ostberg, 2011). Doubts still remain concerning the primary physiological role of ADHs in animals (Hernández-Tobías et al., 2011). CYP2E1 activity also may have deleterious effects. In a model of humanized CYP2E1 transgenic mice (KI), ethanol-induced steatosis, the first step in alcoholic liver disease (ALD), was restored in the humanized animals, as compared to the KO mice, and oxidative stress in the KI mice was increased as compared to wild type animals (Cederbaum, 2010). Thus, modulation of the expression of CYP2E1 and ADHs might lead to disturbances of cellular homeostasis.

The regulation of ADHs by pollutants has not been studied extensively even though these enzymes are ubiquitously expressed (Attignon et al., 2017; Ishii et al., 2001; Ramirez et al., 2012). There are only few studies of CYP2E1, mainly in animals, regarding the effects of

TCDD (Mejia-Garcia et al., 2013; Schulz et al., 2001; Zordoky and El-Kadi, 2010) or pesticides (Chan et al., 2009; Eraslan et al., 2016; Oropeza-Hernández et al., 2003; Sharma et al., 2013).

In this study, which is part of the European HEALS project on the impact of the exposome on human health, we studied in more detail the effects of the combination of TCDD and α-endosulfan on the expression of ADH4 and CYP2E1 in two human hepatic models, HepaRG and HepG2 cells. We focused on the molecular mechanisms and the transcription factors that are involved in the negative regulation of CYP2E1 and ADH4.

Materials and methods

2.1. Compounds

TCDD (#ED-901, CAS: 1746-01-6) and α-endosulfan (CAS: 959-98-8) were purchased from LGC Standards (France). The AHR antagonist CH-223191 (#C8124, CAS: 301326-22-7) and DMSO (#D4540, CAS: 67-68-5), were obtained from Sigma. AHR agonists 3-methylcholanthrene (3-MC, #44-2388, CAS: 56-49-5) and PCB 126 (PolyChloroBiphenyl 126, #RPC-12, CAS: 57465-28-8) were purchased, respectively, from Supelco (France) and Ultra Scientific (Italy). The siRNAs were obtained from Dharmacon (Thermo Scientific) as ON-TARGET plus SmartPool: control, #D-001810-10-05; AHR, #L-003175-00; ARNT, #L-007207-00; SRC, #L-003175-00; CAR #L-003416-00; PXR #L003415-00; ERα #L-003401-00.

2.2. Cell culture and treatments

The HepaRG cell line was a gift from Dr Guguen-Guillouzo (Rennes, France). It was cultured in complete Williams' E medium and differentiated with 1.5 % DMSO (Aninat et al., 2006; Attignon et al., 2017). For the treatments, HepaRG were trypsinized and seeded at 210 000 cells/cm^2. The human hepatocarcinoma cell line HepG2, obtained from the ATCC (#HB-8065), was cultured as described previously (Magne et al., 2007). Seventy-two hours after the seeding, the differentiated HepaRG cells were treated with TCDD (0.01, 0.1, 1, 3, 10 or 25 nM) or α-endosulfan (0.1, 0.3, 1, 3, 5 or 10 µM) alone or as mixtures for 24 hours, for isolation of mRNA, and 72 hours for protein assays. Treatments with 1µM PCB 126 or 5µM 3-MC also were performed for 24 hours. In some cases, the cells were pretreated with the AHR antagonist, CH-223191 (1 or 10 µM), 1h before the treatment. The differentiated HepaRG

cells also were exposed to TCDD (0.1 to 5 nM), α-endosulfan (1 µM) or the mixture for 8 days.

2.3. siRNA transfection

Transfection of differentiated HepaRG cells with siRNAs (against AHR, ARNT, c-SRC, ERα, CAR or PXR) was performed using reverse-transfection as described previously (Attignon et al., 2017). Seventy-two hours later, the medium was replaced and the cells were exposed or not to 25 nM TCDD, 10 µM α-endosulfan, or the mixture. RNA purification and protein extraction were carried out after 24 and 72 hours of treatment, respectively.

2.4. RNA isolation and RT-qPCR

The isolation of RNA from HepaRG and HepG2 cells, reverse transcription, and qPCR analyses were performed as described previously (Attignon et al., 2017). The oligonucleotide sequences (Table 1) were designed using the OLIGO Explorer software or were found in the literature and were purchased from Eurogentec (France). The relative amounts of mRNA were estimated by the delta–delta Ct method (Livak and Schmittgen, 2001) using RPL13A as the reference gene. The variance from the replicate Ct values was carried through to the final calculation of relative quantities using standard propagation of error methods.

2.5. Immunoblotting

Cells were scraped into RIPA buffer as described previously (Attignon et al., 2017). Total proteins (7.5 µg for CYP2E1 and 15 µg for ADH4, AhR, ARNT and PXR) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated for 1 hour at room temperature with blocking buffer and then overnight at 4°C

with the primary antibody. The references for the antibodies and the conditions for western-blotting are given in Supplementary Table S1, After the final washes, the signals were quantified using an Odyssey infrared Imager (LI-COR, ScienceTec) or an LAS 4000 Imager (after ECL revelation).

2.6. Statistical analysis

The data are expressed as the mean ± SD of at least three different experiments. Statistical analysis was carried out in R (*agricolae* package) using the Kruskal–Wallis test (nonparametric comparison of k independent series) followed by the Dunn *post-hoc* test. The p values are indicated as: * or # < 0.05, **, ## or $$ p < 0.01, *** or ### p < 0.001.

Results

3.1. The expressions of alcohol metabolism enzymes are decreased by pollutants alone and in mixtures.

Using a non-targeted whole genome transcriptome analysis, we previously showed that the expression of several genes which encode proteins that are involved in alcohol metabolism was drastically decreased in HepaRG cells, following exposure for 30h to a combination of 25 nM TCDD and 10 µM α-endosulfan (Ambolet-Camoit et al., 2015). These doses are those commonly found in the literature for which effects are observed but which do not result in significant cytotoxicity. Since these doses are relatively high, we have also investigated the effects of lower doses. Now using a PBBK model, we have found that the high doses used in this study correspond to those in highly exposed human populations (Leblanc et al., under review).

When HepaRG cells were exposed for 24h (for mRNA analysis) and 72h (for protein analysis) to either 25 nM TCDD and 10 µM α-endosulfan, separately, or to their mixture, the expression of ADH4 and CYP2E1 mRNAs decreased (up to 90% after treatment with the mixture), (Figure 1A), and the protein levels decreased (up to 50-60% with the mixture) (Figure 1B/C). The inhibition of the expression of mRNA was markedly more pronounced with the mixture as compared with either pollutant alone.

We then studied the effect of 24h treatment with lower concentrations of TCDD or α-endosulfan on the expression of CYP2E1 and ADH4 mRNA. CYP2E1 expression was decreased in a dose-dependent manner by both TCDD (Figure 2A) and α-endosulfan (Figure 2B). ADH4 expression was decreased by α-endosulfan (Figure 2C) and by TCDD, as shown previously (Attignon et al., 2017). With mixtures of TCDD and α-endosulfan, the mRNA levels of CYP2E1 and ADH4 decreased up to 75% at the highest concentrations (mixture of 3nM TCDD and 3

µM α-endosulfan) (Figure 2D/E). As expected, the expression of CYP2B6 and CYP3A4 mRNA was increased by α-endosulfan (Supplementary Figure S1), as described elsewhere (Savary et al., 2014). We showed previously a dose-dependent increase of CYP1A1 mRNA by TCDD in HepaRG cells (Attignon et al., 2017).

3.2. Effects of "sub-chronic" exposures of HepaRG to the pollutants

HepaRG cells were exposed for 8 days to lower concentrations of the POPs (0.1 to 5 nM TCDD and 1 µM α-endosulfan alone and in mixtures). We previously observed no toxicity for 5 nM TCDD, 3 or 10 µM α-endosulfan or for their mixtures (Ambolet-Camoit et al., 2015). Although not significant statistically, the amounts of CYP2E1 and ADH4 mRNAs decreased 40% after treatment with 0.1 nM TCDD and about 30% after treatment with 1 µM α-endosulfan (Figure 3A/B). Significant decreases were observed after treatment with all the mixtures, except one. Maximal inhibitions of 75% and 80% were obtained, respectively, for CYP2E1 and ADH4, with the mixture of 5 nM TCDD and 1 µM α-endosulfan (Figure 3A/B). The amounts of protein also were decreased (Supplementary Figure S2A/B) after treatment by the mixtures (TCDD 0.1 and 1 nM / α-endosulfan 1 and 3 µM).

3.3. Effects of the pollutants in the HepG2 hepatic model

We next analyzed the regulation of the expression of CYP2E1 and ADH4 by TCDD and α-endosulfan in another liver model, the human hepatoma cell line HepG2. These cells express AHR but no CAR and little PXR and ERα (Aninat et al., 2006; Fang et al., 2016). The expression of CYP2E1 is low but detectable. The amounts of CYP2E1 and ADH4 mRNAs decreased significantly (40 and 50 %) following exposure of HepG2 cells for 24 hours to either 25 nM TCDD or its mixture with 10 µM α-endosulfan (Figure 3C/D). We verified that

CYP1A1 was increased by TCDD (data not shown). These results confirm the effects of TCDD on the expression of CYP2E1 and ADHs that were obtained in the human liver HepaRG cells. In contrast, 10 µM α-endosulfan had no significant effect on the expression of CYP2E1 and ADH4 (Figure 3C/D) in HepG2 cells. In these cells, CYP2B6 mRNA was not detectable, whereas CYP3A4 mRNA was weakly expressed but not regulated by α-endosulfan (data not shown).

3.4. The effect of TCDD on CYP2E1 is mediated by the AHR genomic signaling pathway

In a previous paper, we showed that the exposure of several hepatic models (HepaRG, HepG2 and human primary hepatocytes) to TCDD decreases the expression of ADHs (ADH1, AHD4 and ADH6). We demonstrated that these regulations were mediated by the AHR/ARNT complex in the HepaRG model (Attignon et al., 2017). To study the role of the AHR, ARNT and SRC in the regulation of the expression of CYP2E1 by TCDD, we first treated the cells with two AHR agonists (PCB 126 and 3-methylcholanthrene). Both decreased the level of CYP2E1 mRNA (Figure 4A). A known AHR antagonist, CH-223191, at a concentration of 10 µM, completely abolished the effects of 3 and 25 nM TCDD. At a concentration of 1 µM, CH-223191 also was able to abolish the effect of 3nM TCDD (Figure 4B). Finally, specific siRNA targeting of AHR, ARNT and SRC mRNAs was used to decrease the expression of the corresponding mRNAs (Attignon et al., 2017). We verified that the levels of AHR and ARNT proteins were decreased (Supplementary Figure S3A/B). As a positive control, the expression of CYP1A1 mRNA was modified as expected in these experiments (siRNA and AHR antagonist, data not shown). siAHR transfection abolished the decrease in expression of CYP2E1 that resulted from exposure of the cells to TCDD (Figure 5A). In the case of siARNT, we observed a significant increase in the basal level of CYP2E1 mRNA in the absence of

TCDD. Following treatment with TCDD (in the presence of siARNT), there was still some decrease (35%) in the level of CYP2E1 mRNA as compared to the absence of TCDD. This decrease in inhibition was significantly different from the 76% decrease of CYP2E1 mRNA following treatment with TCDD (as compared to the absence of TCDD) in the presence of siCtl (Figure 5B). The siSRC had no effect (Figure 5C).

3.5. The effect of α-endosulfan is not mediated by the PXR the CAR or the ERα pathways.

α-endosulfan has been described as acting through the PXR, CAR or ERα pathways. Although PXR, CAR and ERα mRNA expression was decreased effectively by siPXR, siCAR and siERα transfection, respectively (Supplementary Figure S4A/B/C), there was no modification of the regulation of the expression of either CYP2E1 (Figure 6A/B/C) or ADH4 (Figure 6D/E/F) following exposure of cells to 10 µM α-endosulfan. We verified the efficiency of the siRNAs by measuring the mRNA levels of CYP2B6 and CYP3A4, known targets of PXR and CAR (Figure S5A). In addition, the increase level of mRNA levels of CYP2B6 and CYP3A4 by endosulfan were reduced in the presence of siERα (Supplementary Figure S5B/C). We also verified that the amount of PXR protein was decreased (Supplementary Figure S3C) and that the transfection with siAHR or siARNT did not modify the effect of α-endosulfan on CYP2E1 expression (Supplementary Figure S5D).

4. Discussion

In this paper, we report for the first time that the combination of two POPs, TCDD and α-endosulfan, acting *via* different pathways, drastically decreases the amounts of mRNA and, to a lesser extent, the level of proteins of two enzymes involved in ethanol metabolism (ADH4 and CYP2E1). Moreover, in a proteomic study, ADH4 also was found to be decreased by either TCDD alone or by the combination of the two POPs (E. de Oliveira Cacheado, unpublished results). However, we cannot conclude as to an additive or synergistic effect of the combined POPs since this would require more extensive dose response curves (Kortenkamp and Altenburger, 1998).

Since down-regulation of genes is less common than up-regulation, we investigated the molecular mechanisms involved in these inhibitions. We showed that the genomic AHR/ARNT pathway was implicated in the TCDD effect. A ChIP-Seq analysis revealed no interactions between the AHR/ARNT complex and the promoters of CYP2E1 and ADHs in human MCF-7 cells (Lo and Matthews, 2012) and a search for xenobiotic responsive elements (XREs) only showed potential sites far from the transcription start sites of the genes. Therefore, the regulation of these genes by TCDD might be mediated by an intermediate transcriptional mechanism as has been suggested in primary human macrophages (Podechard et al., 2009). Surprisingly, transfection of siARNT increased the basal level of CYP2E1 (Figure 5B). The same result also was found for ADH4 (data not shown). This indicates that, under basal conditions, ARNT represses, directly or indirectly, the expression of these genes. Indeed, ARNT has been described either a co-activator or a co-repressor in estrogen signaling depending on the cell type (Labrecque et al., 2012). Since ARNT is known to bind other partners (Labrecque et al., 2013) one of these complexes could

have a still unknown inhibitory effect on these genes, which is overridden by the siARNT. Alternatively, AhR could bind an endogenous ligand in the absence of treatment, which could induce the formation of the AHR/ARNT transcriptional complex and lead to the expression of a repressor. In this case, TCDD would enhance the expression of this repressor.

Concerning the down-regulation observed with the pesticide, we excluded the implication of the AHR/ARNT pathway. We tested the PXR, CAR and ERα pathways, which are involved in the increased expression of CYP3A4 and CYP2B6 in various cell lines treated with OCPs, including α-endosulfan (Casabar et al., 2006; Coumoul et al., 2002; Lemaire et al., 2006; Savary et al., 2014). Under our experimental conditions, none of the nuclear receptors seemed to be involved in the down-regulation of ADH4 and CYP2E1 by α-endosulfan. In another cell line, HepG2, we only observed an effect with TCDD in accordance with the expression of the xenosensors (Aninat et al., 2006; Fang et al., 2016). In addition, we detected no CYP2B6 and little CYP3A4 which can metabolize endosulfan (Silva and Beauvais, 2010). This suggests that the endosulfan effect in HepaRG cells could be due to a metabolite of the pesticide. Since siRNAs against PXR, CAR and ER had no effect on the inhibition of expression of CYP2E1 and ADH4 by endosulfan, we verified their functionality on the expression of CYP3A4 and CYP2B6 in HepaRG cells. We observed a down-regulation of the levels of mRNA of CYP2B6 by siCAR and of CYP3A4 by siPXR, as expected. In addition, the siERα abolished the up-regulations of CYP3A4 and CYP2B6 by α-endosulfan. Thus, these signaling pathways are not involved in the regulation of CYP2E1 and ADH4. Alternatively, other mechanisms could be involved for these genes. For example, the increased production of reactive oxygen species by a CYP has been linked to a decrease of other CYPs such as CYP2E1 (Morel et al., 2000). Also, one study described that α-endosulfan could be a weak agonist of retinoic acid receptors (RARβ and γ) in HeLa cells transfected with a reporter

plasmid (Lemaire et al., 2005). A treatment with 15 µM endosulfan (mixture of the α- and β-isomers) also increased the expression of CYP26A1, a target gene of RAR, in HepG2 cells (Gandhi et al., 2015). In the HepaRG model, the amount of CYP26A1 mRNA was not modified after treatment with 10 µM α-endosulfan (Ambolet-Camoit et al., 2015). This discrepancy could be due to the use of different cellular models and/or to the isomers used.

Several studies have investigated the role of ADHs in diverse metabolismic pathways. In ADH knockout (KO) mice a reduced production of retinoic acid following vitamin A (all-*trans*-retinol) administration is observed (Deltour et al., 1999) and vitamin A is toxic due to the lack of metabolism (Molotkov and Duester, 2003). In humans, ADH1 and 4 play a major role in ethanol and retinol metabolism (Edenberg, 2000; Parés et al., 2008). Recently, a study showed that ADH1 was decreased in patients with alcoholic liver disease (ALD) (Kumar et al., 2016), but it is not known if this decrease is a cause or a consequence of the ALD. Moreover, poor prognosis for hepatocarcinoma has been linked with low levels of expression of ADH4 (Wei et al., 2012).

To date, CYP2E1 KO has not been associated with any pathology (Lu et al., 2008; Wang et al., 2016). In these KO mice, oxidative stress and hepatic steatosis were blunted after ingestion of ethanol (Lu et al., 2008) or in a high-fat diet-induced obesity (Zong et al., 2012). The protective effect is likely linked to reduced production of reactive oxygen species. In humans, an increased amount of CYP2E1 mRNA has been found in patients with steatosis, non-alcoholic liver steatohepatitis and ALD (Aljomah et al., 2015; Song et al., 2015). In a context of alcohol consumption, the inhibition of the hepatic alcohol metabolizing enzymes, which protects the liver, could promote the formation of cytotoxic fatty acid ethyl esters through a non-oxidative metabolism of ethanol (Zelner et al., 2013). The decrease in CYP2E1 and ADH activities likely reduces the first-pass metabolism of ethanol. This may increase the

toxic effects due to impregnation of extra-hepatic tissues, in particular the brain, and potentially lead to increased neurotoxicity (Hernández et al., 2016).

In conclusion, this *in vitro* study is the first to describe the inhibition of CYP2E1 expression by two POPs through independent pathways, one involving AHR/ARNT for TCDD and another one, still unknown, for α-endosulfan. It has been suggested previously that the increased expression of CYP1A1 by an AhR ligand (Benzo[a]pyrene) could inhibit the expression of other CYP members such as CYP2E1 through a repressive cross-regulation (Morel et al., 2000). In addition, the concerted decrease of the expression of CYP2E1 and ADH4, two alcohol metabolizing enzymes, after exposure of human hepatic cells to a combination of POPs suggests that this combination could have more deleterious effects than a single pollutant on the liver and a detrimental impact on several metabolic pathways. These results could help to account for the association between the increased incidence of liver diseases and POP levels in several epidemiological studies (Lee et al., 2014; Magliano et al., 2014; Taylor et al., 2013; Wahlang et al., 2013). They also could contribute to the establishment of safety levels for human exposure to mixtures of POPs, which are important in the context of diets linked to chronic liver diseases (for example alcohol consumption or high-fat diet).

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Legends to the Figures

Figure 1. Effect of the exposure of HepaRG cells to TCDD, α-endosulfan or the mixture on the amounts of ADH4 and CYP2E1 mRNA and protein. The relative (as compared to non-exposed cells) levels of CYP2E1 and ADH4 mRNA (A) and protein (B/C) were measured after 24 and 72h, respectively, of exposure to the POPs (25 nM TCDD, 10 µM α-endosulfan or their mixture). The levels of significance of the fold-change as compared to the control (*) or between the mixture and each pollutant alone (#) are as indicated in the Methods.

Figure 2. Dose response curves of TCDD, α-endosulfan and the mixture on the levels of CYP2E1 and ADH4 mRNAs. The relative (as compared to non-exposed cells) levels of CYP2E1 mRNA were measured after 24h of exposure to increasing concentrations of TCDD (A), α-endosulfan (B) or their mixture (D). The levels of ADH4 mRNA were measured with increasing concentrations of α-endosulfan (C) or their mixture (E). The levels of significance of the fold-change compared to the control (*) are as indicated in the Methods.

Figure 3. Effects of "sub-chronic" exposures of HepaRG cells and of an acute exposure in HepG2 cells. The relative levels of CYP2E1 (A) and ADH4 (B) mRNAs were measured after 8 days of exposure of HepaRG cells to TCDD (0.1, 0.2, 0.5, 1 or 5 nM) or 1 µM α-endosulfan or their mixtures. The control value without pollutant was used as the basal level (1). The relative amounts of CYP2E1 (C) and ADH4 (D) mRNAs were measured after 24h of treatment of HepG2 cells with 25 nM TCDD, 10 µM α-endosulfan or their mixture. The levels of significance of the fold-change as compared to the basal values (*) are as indicated in the Methods. ns: not significant statistically.

Figure 4. Effects of AHR agonists and of an antagonist CH-223191 on the level of CYP2E1 mRNA. HepaRG cells were treated with PCB 126 (1 µM) or 3-MC (5 µM) for 24h (A). Cells were pre-treated for 1h with the AHR antagonist CH-223191 (1 or 10 µM) prior to a 24h exposure to 3 or 25 nM TCDD (B). Then, the relative (as compared to non-exposed cells) levels of CYP2E1 were measured. The levels of significance of the fold-change as compared to the Control (*) or to the TCDD treatment alone (#) are as indicated in the Methods. ns: not significant statistically.

Figure 5. Effects of AHR, ARNT and SRC silencing on the expression of CYP2E1 mRNA. HepaRG cells were transfected for 72h with either a control siRNA (siCtl) or siRNA directed against AHR (A), ARNT (B) or SRC (C), respectively. Then, the cells were treated or not with 25 nM TCDD for 24h and the relative levels of CYP2E1 mRNA were measured. The siCtl value without TCDD was used as the basal level (1). The levels of significance of the fold-change as compared to the siCtl (*), siCtl + TCDD (#) or siARNT ($) are as indicated in the Methods.

Figure 6. Effects of PXR, CAR and ERα silencing on the expression of ADH4 and CYP2E1 mRNAs. After transfection for 72h with either a control siRNA (siCtl) or siRNA directed against PXR (A/D) or CAR (B/E) or ERα (C/F), HepaRG cells were treated or not with 10 µM α-endosulfan for 24h. Then, the relative levels of CYP2E1 (A/B/C) and ADH4 mRNAs (D/E/F) were measured. The siCtl value without the pesticide was used as the basal level (1) for each gene. The levels of significance of the fold-change as compared to the siCtl (*) are as indicated in the Methods.

Legends to the Supplementary Figures

Figure S1. Dose response curves for the effect of α-endosulfan on the levels of CYP2B6 and CYP3A4 mRNAs. The relative levels of CYP2B6 (A) and CYP3A4 (B) mRNA were measured after 24h of exposure to increasing concentrations of α-endosulfan as compared to non-exposed cells. The levels of significance of the fold-change as compared to the control (*) are as indicated in the Methods.

Figure S2. Effects on the levels of CYP2E1 and ADH4 proteins following "sub-chronic" exposures of HepaRG cells to POPs.

The relative levels of CYP2E1 (A) and ADH4 (B) proteins were measured after 8 days of exposure of HepaRG cells to TCDD (0.1 or 1nM) or α-endosulfan (1 or 3 μM) or their mixtures. The control values without pollutants were used as the basal levels normalized to β-actin (1) (n=1).

Figure S3. Amounts of AHR, ARNT and PXR proteins after siRNA transfection. The amounts of proteins were measured after transfection with either a control siRNA (siCtl) or siRNA directed against AHR (A), ARNT (B) or PXR (C) for 96h. The control value with siCtl was used as the basal level normalized to β-actin (1) (n=1).

Figure S4. Levels of PXR, CAR and ERα mRNAs **after siRNA transfection.** After transfection for 72h with either a control siRNA (siCtl) or siRNA directed against PXR (A), CAR (B) or ERα (C), HepaRG cells were treated or not with 10 μM α-endosulfan for 24h. The relative levels

of the respective mRNAs were measured. The siCtl value without the pesticide was used as the basal level (1) for each gene. The levels of significance of the fold-change as compared to the siCtl (*) are as indicated in the Methods.

Figure S5. Levels of CYP2B6, CYP3A4 and CYP2E1 mRNAs after siRNA transfection. After transfection for 72h (mRNA) with either a control siRNA (siCtl) or siRNA directed against ERα, HepaRG cells were treated or not with 10 µM α-endosulfan for 24h. The relative levels of CYP2B6 (A) and CYP3A4 (B) mRNAs were measured. The siCtl value without the pesticide was used as the basal level (1) for each gene. C : After transfection for 72h (mRNA) with either a control siRNA (siCtl) or siRNA directed against CAR or PXR, the relative levels of CYP2B6 and CYP3A4 mRNAs were measured. The siCtl value was used as the basal level (1) for each gene (n=1). D: After transfection for 72h (mRNA) with either a control siRNA (siCtl) or siRNA directed against AHR or ARNT, and treatment or not with 10 µM α-endosulfan for 24h, the relative level of CYP2E1 mRNA was measured. The levels of significance of the fold-change as compared to the siCtl (*), siCtl+endo (#) or siARNT ($) are as indicated in the Methods (n=2-4).

Table 1

Primer sequences for RT-qPCR assays

gene	Forward Primer (5’ – 3’)	Reverse Primer (5’ – 3’)
ADH4	GCATTGAAGAGGTTGAAGTAGC	GATAACAGTGGCATCAGTATGG
AHR	ACATCACCTACGCCAGTCGC	TCTATGCCGCTTGGAAGGAT
ARNT	ACCAGCCACAGTCTGAATG	TCTCCTTGAGCCCATACAC
CAR	AGACCGACCTGGAGTTACC	CTGGATGTGCTGGATTTG
CYP1A1	GGTCAAGGAGCACTACAAAACC	TGGACATTGGCGTTCTCAT
CYP2B6	GAAAAACCAGACGCCTTCAATCCT	CCAAGACAAATCCGCTTCCCTAA
CYP2E1	ACTATGGGATGGGGAAACAG	GAGGATGTCGGCTATGACG
CYP3A4	GATGGCTCTCATCCCAGACTT	AGTCCATGTGAATGGGTTCC
ERα	ATCCTACCAGACCCTTCAGTG	CAGACGAGACCAATCATCAG
PXR	CCAAGCGACCAAGGATG	TCAGGAAGCGAACAAACG
RPL13A	AAGGTCGTGCGTCTGAAG	GAGTCCGTGGGTCTTGAG
c-SRC	CTGAGGAGTGGTATTTTGGC	GGCGTGTTTGGAGTAGTAGG

Figure 1

2-column width

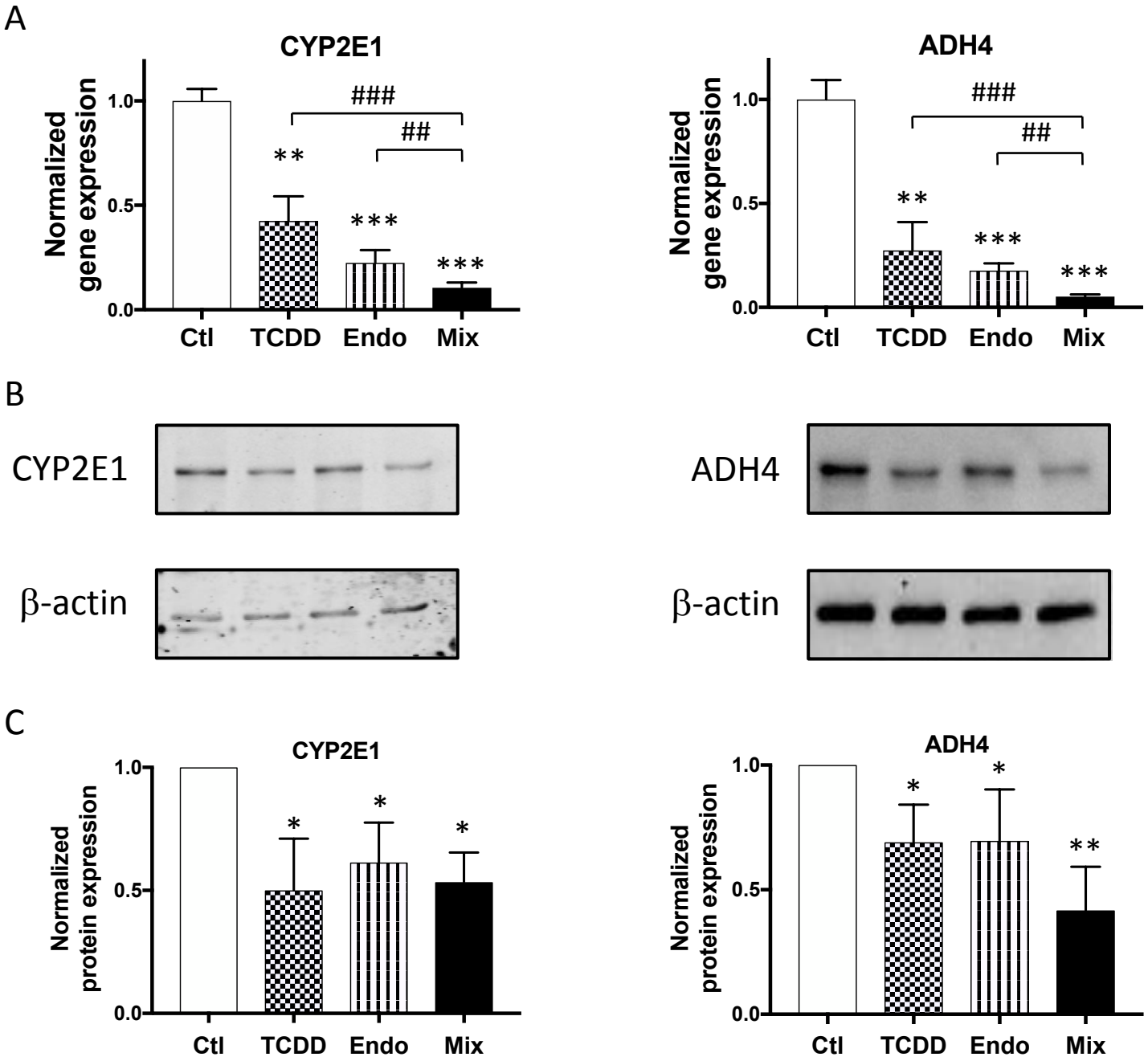

Figure 2

2-column width

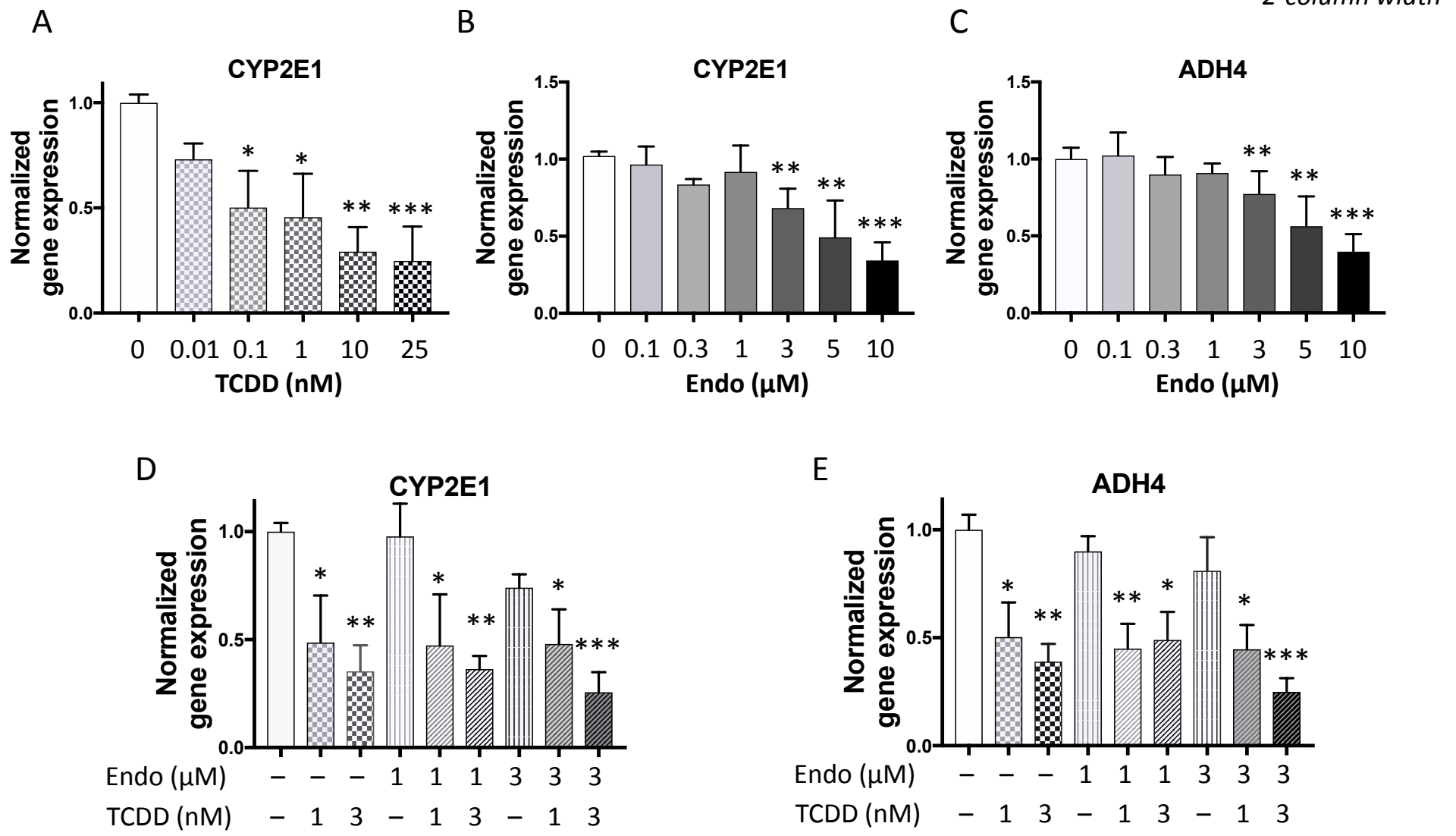

Figure 3

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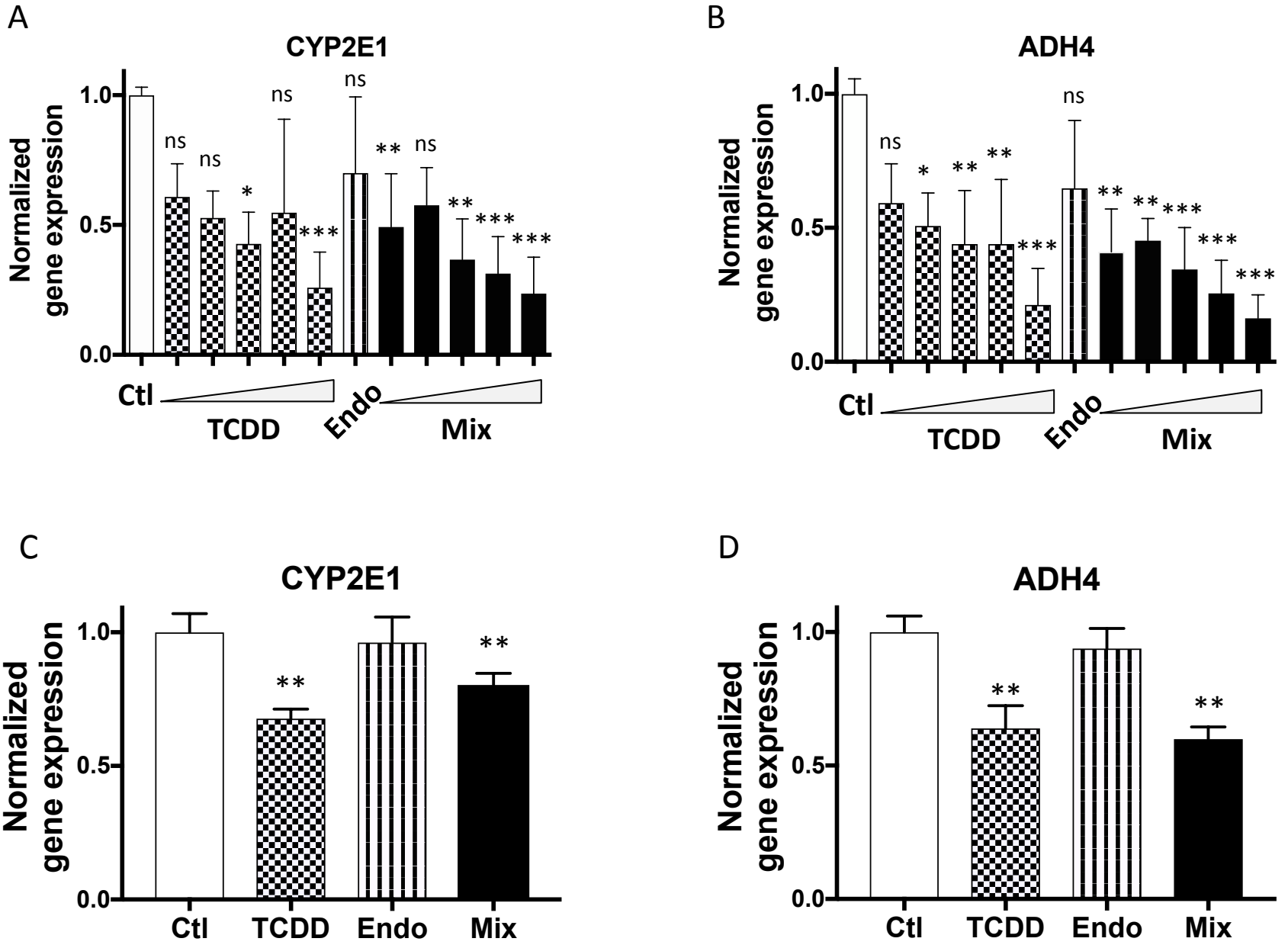

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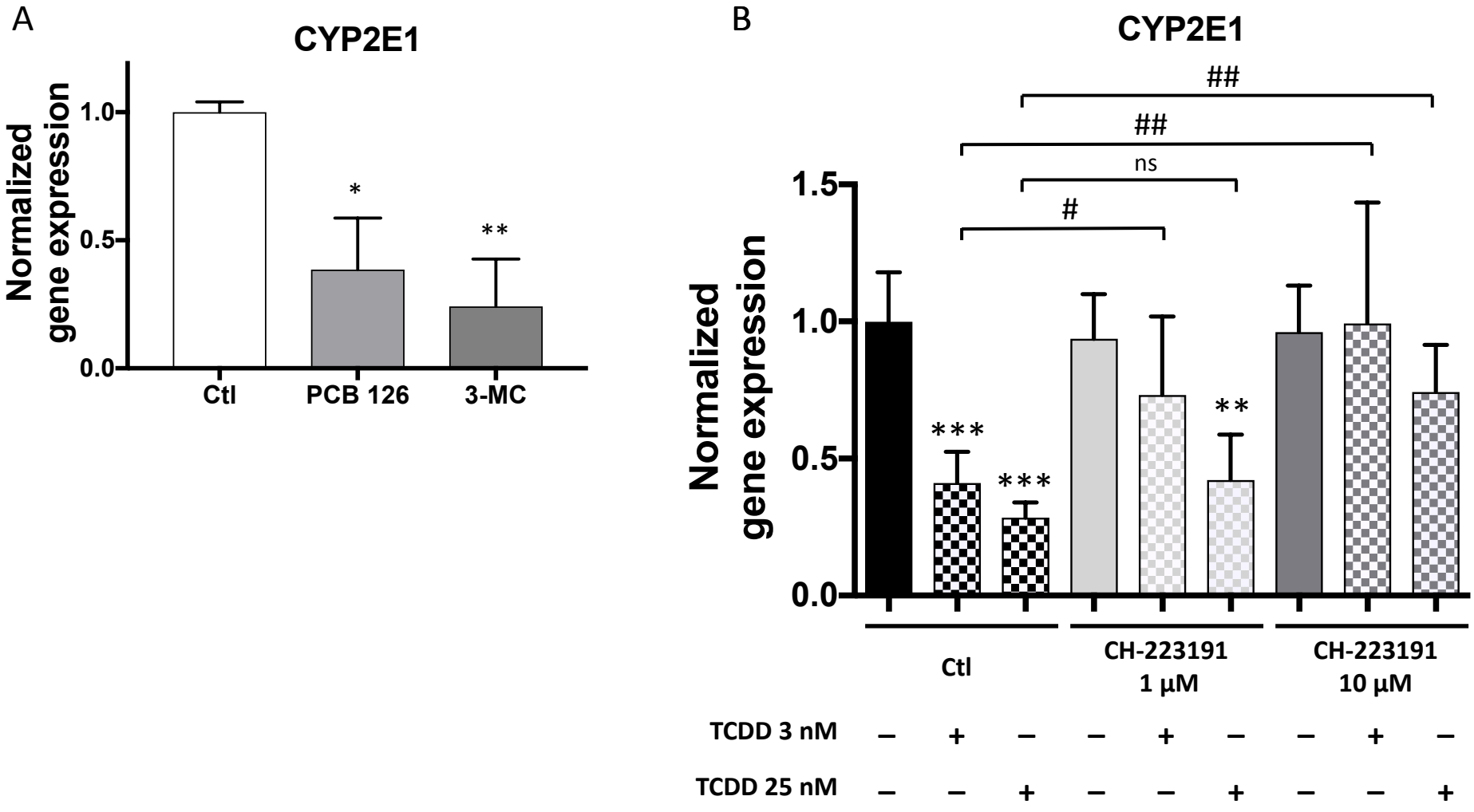

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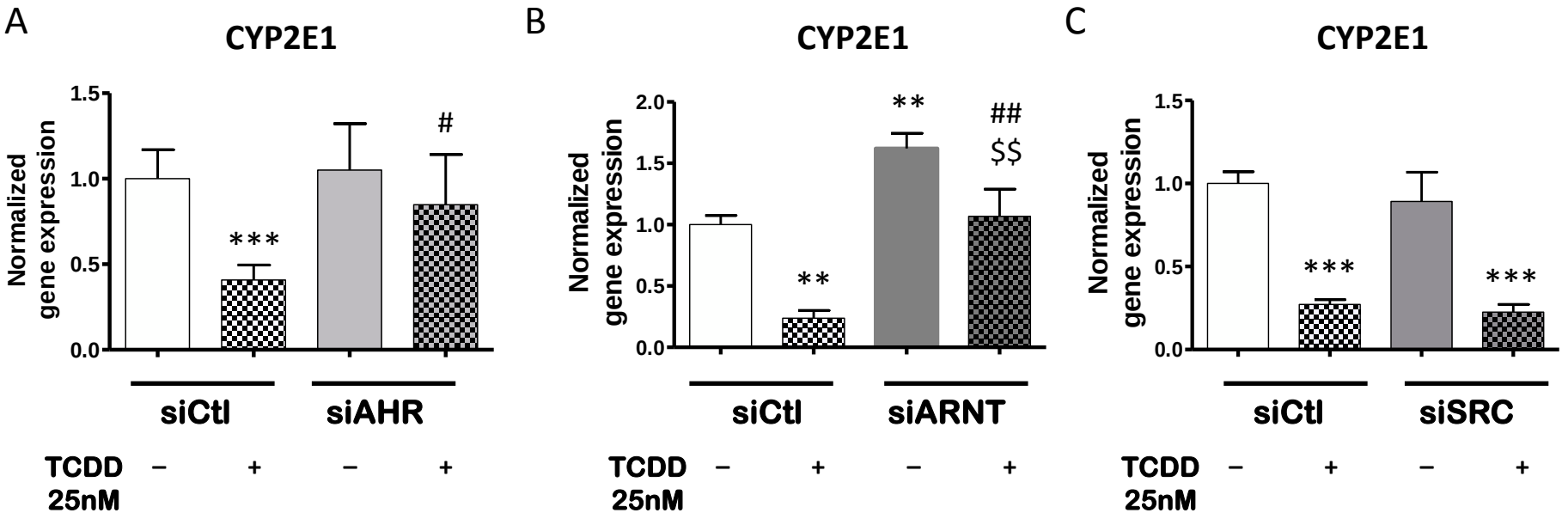

Figure 6

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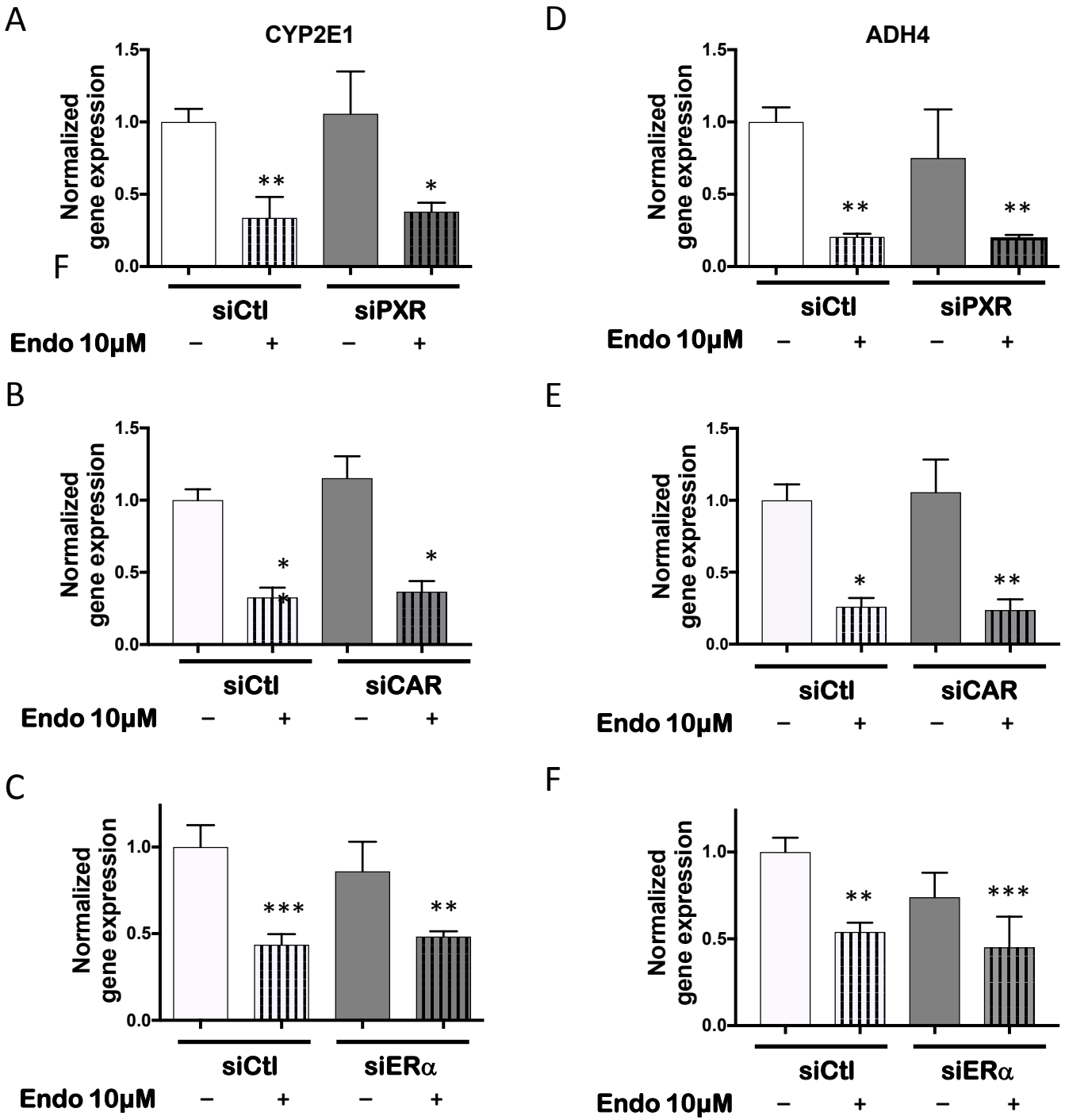

Supplementary Material

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Highlights

- *In vitro* exposure to POPs down-regulates the expression of alcohol metabolizing enzymes
- TCDD and/or α-endosulfan inhibit the expression of CYP2E1 and ADH4 in HepaRG cells
- TCCD acts through the AHR/ARNT genomic pathway
- The PXR, CAR and ERα pathways are not involved in the effect of α-endosulfan

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