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## **Di-(2-ethylhexyl)-phthalate (DEHP) activates the Constitutive Androstane Receptor (CAR): a novel signalling pathway sensitive to phthalates**

Alexandre Eveillard, Laila Mselli-Lakhal, Ariane Mogha, Frédéric Lasserre, Arnaud Polizzi, Jean-Marc Pascussi, Hervé Guillou, Pascal G.P. Martin, Thierry Pineau

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1 **Di-(2-ethylhexyl)-phthalate (DEHP) activates the Constitutive Androstane**  
2 **Receptor (CAR): a novel signalling pathway sensitive to phthalates.**  
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**Abstract**

Di-(2-ethylhexyl)-phthalate (DEHP), a widely used plasticizer, is detected in consumer's body fluids. Contamination occurs through environmental and food chain sources. In mouse liver, DEHP activates the peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) and regulates the expression of its target genes. Several *in vitro* investigations support the simultaneous recruitment of additional nuclear receptor pathways.

We investigated, *in vivo*, the hepatic impact of low doses of DEHP on PPAR $\alpha$  activation, and the putative activation of additional signalling pathways. Wild-type and PPAR $\alpha$ -deficient mice were exposed to different doses of DEHP. Gene expression profiling delineated the role of PPAR $\alpha$  and revealed a PPAR $\alpha$ -independent regulation of several prototypic Constitutive Androstane Receptor (CAR) target genes. Thus, we developed an original hepatic cell line expressing CAR to investigate its activation by DEHP. By means of a pharmacological inhibitor or CAR-targeting shRNAs, we established that CAR is required for the effect of DEHP on Cyp2b10, a recognized CAR target gene. Moreover, DEHP dose-dependently induced CYP2B6 in human primary hepatocyte cultures.

This finding demonstrates that CAR also represents a transcriptional regulator sensitive to phthalates. CAR-mediated effects of DEHP provide a new rationale for most endpoints of phthalates toxicity described previously, including endocrine disruption, hepatocarcinogenesis and the metabolic syndrome.

## 1. Introduction

Phthalates represent a leading class of chemical congeners which are used in a broad range of industrial applications including plasticized polyvinyl chloride [PVC], cosmetics, residential construction, automotive industries and medical products [1, 2]. Since phthalates are not chemically bound to the products, they can leak, migrate or evaporate mostly from PVC-containing goods, resulting in significant environmental contamination [3] and human exposure [4-7]. Di-(2-ethylhexyl)-phthalate (DEHP) is a widely used congener for which dietary exposure (food processing, packaging) likely represents the main source of contamination for the general population [5]. The highest levels of exposure have been reported in specific populations such as patients and neonates exposed to PVC medical devices [8] or PVC factory workers [9].

An important concern about DEHP relates to its potential to act as a nongenotoxic hepatocarcinogen in rodent species [10]. Initial experiments implicated PPAR $\alpha$ -dependent mechanisms and suggested a controversial low relevance to humans [11, 12]. However, while PPAR $\alpha$ -null mice (SV129 background) develop liver neoplasms under DEHP exposure [13] the mechanisms implicated may be slightly different from those elicited in wild-type mice [14]. Thus, this supports the need for more investigations to elucidate the mechanism leading to carcinogenesis.

Through experimental and epidemiology studies, evidence has accumulated for the association of other detrimental health effects with phthalate exposure. In mammals, including humans, phthalate exposure is associated with endocrine disruption [15-17] and metabolic disorders [18, 19]. In rodents, *in utero* exposure to phthalates results in developmental and reproductive defects similar to those termed *testicular dysgenesis syndrome* in humans [20-22]. Furthermore, an association between phthalate exposure and subtle defects in genital tract development of male infants has been reported [23]. The underlying mechanisms may involve antiandrogenic or proestrogenic properties of phthalates that could disturb the gonadal

1 development in the embryo through the modification of androgens bioavailability [24]. Sustained  
2  
3 exposure to phthalates through adulthood could also contribute to decreased testosterone levels  
4  
5 [9] or semen quality [25].  
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7  
8 Recently, two independent cross sectional studies of the National Health And Nutrition  
9  
10 Examination Survey [NHANES, 1999-2002] have provided consistent conclusions which  
11  
12 associate several urinary phthalates metabolites with key features of the metabolic syndrome  
13  
14 (body mass index, abdominal adiposity and insulin resistance) [18, 19]. Based on several studies,  
15  
16 it has been suggested that the onset of the metabolic syndrome could be favoured by prolonged  
17  
18 exposure to low concentrations of environmental pollutants, including phthalates, which are  
19  
20 collectively referred to as *environmental obesogens* [18, 26, 27]. However, the mechanisms  
21  
22 involved in these new aspects of phthalate metabolic impact remain poorly understood. At the  
23  
24 molecular level, the activation of the three isoforms of the peroxisome proliferator-activated  
25  
26 receptor (PPAR,  $\alpha$ ,  $\beta$ ,  $\gamma$ ) by phthalate metabolites and the resulting metabolic consequences have  
27  
28 been extensively documented [28]. In adipose cells, mono-ethylhexyl- phthalate (MEHP), a  
29  
30 potent metabolite of DEHP, activates PPAR $\gamma$  (Nr1c3) and promotes adipogenesis [29]. In liver,  
31  
32 another metabolically relevant organ, phthalates activate PPAR $\alpha$  (Nr1c1) and behave as  
33  
34 prototypical peroxisome proliferators (PP) [30], with rodents being more responsive than  
35  
36 primates [31]. Gene expression profiling studies have further confirmed the striking over  
37  
38 representation of PP-responding genes in the liver of rodents exposed to DEHP [32, 33]. Aside  
39  
40 from PPAR $\alpha$ -dependent effects, phthalates were shown to activate the murine and human  
41  
42 pregnane X nuclear receptor (PXR, Nr1i2), thus increasing the transcription of cytochrome P450  
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44 3A (Cyp3A) [34, 35].  
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54 The main objective of this study was to investigate the influence of DEHP on the network  
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56 of hepatic nuclear receptors, while leaving aside the PPAR $\alpha$ -dependent response, which has  
57  
58 already been well established. Using gene expression profiles dedicated to NR pathways, we  
59  
60

1 identified the Constitutive Androstane Receptor (CAR, Nr1i3) as a putative phthalate target.  
2  
3 Then we developed specific recombinant cellular tools to establish that phthalates activate the  
4  
5 CAR signalling pathway. Finally, we showed that DEHP dose-dependently induce the expression  
6  
7 of the CAR target gene CYP2B6 in primary human hepatocytes. This original finding is  
8  
9 discussed in view of the crucial role played by the liver in controlling energy homeostasis and the  
10  
11 recent findings which associate phthalate exposure to the onset of the metabolic syndrome in men  
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13 [18, 19].  
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## 20 **2. Materials and Methods**

### 21 **2.1. Materials**

22 Di-(2-ethylhexyl)-phthalate (DEHP) (CAS No. 117-81-7,  $\geq 97\%$  purity),  $5\alpha$ -androst-16-en-3 $\alpha$ -ol  
23  
24 (androst-enol), 1,4-bis[2-(3,5-Dichloropyridyloxy)]benzene (TCPOBOP), dimethylsulfoxide  
25  
26 (DMSO), dexamethasone, geneticin and sodium butyrate were purchased from Sigma (Lyon,  
27  
28 France). Cell culture medium (DMEM), Fetal Bovine Serum (FBS), glutamine, penicillin and  
29  
30 streptomycin, trypsin, lipofectamine 2000 and sodium pyruvate were purchased from Invitrogen  
31  
32 (Cergy Pontoise, France). Mono-(2-ethylhexyl) phthalate (MEHP) was kindly provided by Dr  
33  
34 Jegou (Inserm-U625, Rennes, France).  
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40

### 41 **2.2. Animals and maintenance**

42 Male PPAR $\alpha$ -null mice on a C57BL/6J genetic background [36, 37], and aged-matched wild-  
43  
44 type counterparts (Charles River, Les Oncins, France; 4 weeks acclimatization) were housed in  
45  
46 polycarbonate cages at 22 $\pm$ 2 $^{\circ}$ C on a 12 hour light/dark cycle and allowed free access to water and  
47  
48 food. *In vivo* studies were conducted under European Union Guidelines for the use and care of  
49  
50 laboratory animals and were approved by an independent ethics committee.  
51  
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55

### 56 **2.3. Experimental design, body fluids and organs sampling**

1 At the age of 18 - 22 weeks with an average initial body weight of 26.6 g ( $\pm 1.8$ ; SD), mice from  
2  
3 both genotypes were randomly assigned into six groups (n=10 per group). DEHP, in solution into  
4  
5 corn oil, was administered for 21 days by daily gavages (corn oil control group, 20 or 200  
6  
7 mg/kg/day of DEHP). Blood samples were collected at the retro-orbital sinus with heparin-coated  
8  
9 capillaries on three days (-1, 10 and 21). Plasma was prepared by centrifugation at 16000 g, for  
10  
11 10 min and kept at  $-80^{\circ}\text{C}$ . At day 21, mice were euthanized by cervical dislocation and the liver  
12  
13 was removed, weighed, dissected, snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until being  
14  
15 used for transcriptomic analysis.  
16  
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19

#### 20 **2.4. Transcriptomic analyses**

21  
22 Organ-specific transcriptomic profiles were obtained using a nuclear receptors-dedicated cDNA  
23  
24 macroarray (INRArray 01.4) previously developed and validated [38, 39]; full list of 320 cDNA  
25  
26 probes available at <http://www.inra.fr/internet/Centres/toulouse/pharmacologie/pharmacomoleculaire/technologie/inrarray-01-4.html>. Total hepatic RNA was extracted with TRIzol®  
27  
28 reagent (Invitrogen, Cergy Pontoise, France) according to the manufacturer's protocol. RNA  
29  
30 integrity was controlled on a Bioanalyzer 2100 (Agilent, Massy, France) and assayed at 260 nm  
31  
32 on a Lambda 650 spectrophotometer (Perkin Elmer, Toulouse, France). RNA labelling and  
33  
34 hybridization to INRArray 01.4 were performed as previously described [39]. The raw and  
35  
36 processed data are available in the Gene Expression Omnibus (GEO) database [40] under the  
37  
38 accession number GSE14920.  
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46 For real-time quantitative PCR (Q-PCR), total RNA samples (2 $\mu\text{g}$ ) were reverse-transcribed  
47  
48 using SuperScript™ II reverse transcriptase (Invitrogen, Cergy Pontoise, France). Primers for  
49  
50 SYBR Green assays are presented in Table 1. Amplifications were performed on an ABI Prism  
51  
52 7000 (Applied Biosystems, Courtaboeuf, France). Q-PCR data were normalized by TATA-box  
53  
54 binding protein (TBP) mRNA levels and analyzed with DART-PCR [41].  
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#### 58 **2.5. Statistical analysis of transcriptomic data**



1 Data were analyzed using R ([www.r-project.org](http://www.r-project.org)) with various packages from Bioconductor  
2  
3 ([www.bioconductor.org](http://www.bioconductor.org)). Macroarray data pre-processing (filtering and normalization) and  
4  
5 quality controls were described previously [42]. Differential effects were analyzed by ANOVA  
6  
7 followed by a Student *t* test with a pooled variance estimate. The Benjamini-Hochberg procedure  
8  
9 was used to control the false discovery rate at 10%. Principal component analysis (PCA), an  
10  
11 unsupervised exploratory multidimensional analysis, was used to identify the main effects on the  
12  
13 transcriptomic data set.  
14  
15

## 16 17 **2.6. DNA and shRNA constructs**

18  
19 The pCR3-mCAR construct expressing the murine CAR [43] and the pEGFP-CAR [44] plasmid  
20  
21 expressing a fusion protein between the green fluorescent protein (GFP) and the murine CAR  
22  
23 were kindly provided by Dr. M Negishi. The pLKO-SH1012, pLKO-SH767, pLKO-SH1134,  
24  
25 pLKO-SH648 and pLKO-SH504 HIV-1-derived lentiviral constructs (Sigma, Lyon, France) were  
26  
27 used to knockdown the expression of CAR. The pLVTHM construct [45] is a HIV1-derived  
28  
29 lentiviral vector carrying the GFP gene under the control of the Elongation Factor 1- $\alpha$  promoter.  
30  
31 The packaging system is composed of the psPAX2 and pMD2.G plasmids  
32  
33 (<http://tronolab.epfl.ch/>). All constructs were verified by sequence analysis.  
34  
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37

## 38 39 **2.7. Cell culture and transfections**

40  
41 JWZ murine hepatic cells, also referred to as MuSH immortalized hepatocytes [46], were kindly  
42  
43 provided by Dr. JP Gray. 293FT cells were purchased from Invitrogen (Cergy Pontoise, France).  
44  
45 Both cell lines were cultured in DMEM supplemented with L-glutamine (2mM), penicillin (100  
46  
47 IU/ml), streptomycin (100 $\mu$ g/ml), and BFS (10%). The culture medium of JWZ cells was  
48  
49 supplemented with dexamethasone (1 $\mu$ M) and those of 293FT cells was supplemented with  
50  
51 sodium pyruvate (1mM) and non-essential amino acids (100 $\mu$ M, Invitrogen, Cergy Pontoise,  
52  
53 France). The pCR3-mCAR plasmid was transfected into the JWZ cells using lipofectamine 2000  
54  
55 according to the manufacturer's protocol. After 48 hours, the transfected cells were submitted to  
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1 geneticin selection for 10-15 days. Individual resistant clones were harvested, amplified and  
2  
3 cryopreserved before being tested for CAR transcript expression using Q-PCR.  
4

5 The pLVTHM and the pEGFP-CAR plasmids were transfected into the JWZ cells using  
6 lipofectamine 2000. After 48 hours, the cells were transferred to LabTek chambered  
7  
8 coverslips (Nunc) for fluorescence analysis.  
9  
10

## 11 **2.8. Fluorescence analysis**

12 Cells adherent to the coverslips were fixed with 4% (w/v) paraformaldehyde (Sigma, Lyon  
13 France) in PBS. Coverslips were then mounted onto slides using prolong gold antifade reagent  
14  
15 (Invitrogen, Cergy Pontoise, France) and visualized under a ZEISS LSM 510 laser scanning  
16  
17 confocal microscope.  
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## 23 **2.9. Lentiviral ShRNA vectors production and JWZ-CAR cell transduction**

24 To produce the lentiviral particles, 293FT cells were cotransfected in suspension with the  
25 appropriate lentiviral-shRNA or control vector and the packaging constructs using lipofectamine  
26  
27 2000. After 24 hours the culture medium was replaced with fresh medium containing sodium  
28  
29 butyrate (10mM) known to improve vector titers. Viral supernatants were harvested 48 hours  
30  
31 after transfection, filtered (0.45  $\mu$ m) and titrated on 293FT cells by serial dilutions and  
32  
33 fluorescence-activated cell sorter (FACS) analysis for the ratio of GFP positive cells. The vectors  
34  
35 harbouring the puromycin-resistance gene in place of the GFP gene were titrated according to  
36  
37 Sigma recommendations. Viral titers of  $\sim 10^5$  transforming units (TU)/ml were obtained. To  
38  
39 isolate stable shRNA cell lines, JWZ-CAR (JWZ cells expressing active CAR) were infected  
40  
41 with the viral vector stocks at a multiplicity of infection (MOI) of 1. Following puromycin  
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43 selection (10-15 days) CAR and Cyp2b10 transcripts expression were measured by Q-PCR.  
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## 53 **2.10. Primary culture of human hepatocytes**

54 Human hepatocytes were prepared and cultured according to the previously published procedure  
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56 [47]. CYP2B6 transcript expression was measured by Q-PCR.  
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### 3. Results

#### 3.1. Hepatic impact of DEHP: gene profiling in wild-type and PPAR $\alpha$ -deficient mice

To evaluate the modulation by DEHP of hepatic NR pathways other than PPAR $\alpha$ , we used a NR-dedicated cDNA macroarray (INRArray01.4) to profile hepatic gene expression in wild-type and PPAR $\alpha$ -deficient mouse liver orally exposed to DEHP. We analyzed the hepatic gene expression profile of 60 mice (30 of each genotype, n=10 per group) which were treated for 3 weeks with 0, 20 or 200 mg/kg/day of DEHP. Noticeably, a 20 mg/kg/day dose (corresponding to a diet contamination of ~0.02%) is within the low range of doses inducing liver tumors during long-term exposures in rodents [13, 48]. 284 genes with measurable expression levels were initially retained for further analysis. One third (88 genes), displayed a significant regulation in at least one of the experimental conditions (genotype or treatment effects, Table 2). These regulated genes were assigned to 8 major biological functions (Table 2). Fatty acid homeostasis and xenobiotic metabolism were the most represented pathways. 56 transcripts were differentially expressed between wild-type and PPAR $\alpha$ -deficient control mice. Interestingly, while DEHP (high dose) influenced 49 transcripts in wild type mice, 16 modulations were recorded in PPAR $\alpha$ -deficient livers, and the following 4 regulations occurred in both genotypes: acyl-Coenzyme A oxidase1 (Acox1), aldehyde deshydrogenase family 1 subfamily 1a1 (Aldh1a1), aminolevulinic acid synthase 1 (Alas1) and cytochrome P450 2c29 (Cyp2c29). The 88 transcripts (Table 2) and the 60 samples were subjected to Principal component analysis (PCA) to identify patterns of coregulations that failed to be identified through gene-by-gene statistical tests. The first plane displayed more than 60% of the total variance within the dataset (Fig.1). Wild-type and PPAR $\alpha$ -deficient samples were clearly separated along the first principal component (PC1, Fig.1A, squares versus circles). Additionally, the livers from wild-type mice exposed to DEHP (200 mg/kg/day) were also separated from controls along this axis (Fig.1A, black squares). Thus,

1 PC1 captured a constitutive genotype effect and a PPAR $\alpha$ -dependent effect of DEHP, both  
2 effects affecting similar genes. These genes are the longer vectors displaying a low angle with  
3 PC1 on the loading plot in Fig.1B, such as Cyp4a10, Ech1, Scd1, Angptl4, Cyp4a14, Cdkn1,  
4  
5  
6  
7  
8 Acox, Peci, Apoa4 or Slco1a1. Most of these genes are well-known PPAR $\alpha$  targets [49].  
9  
10 Remarkably, when considering PC2 alone, the impact of DEHP exposure (200 mg/kg/day) was  
11 noticed for both genotypes (overall position of black circles and squares within the upper half of  
12 PC2). The loading plot (Fig.1B) indicated that the following transcripts were co-ordinately  
13 increased by DEHP in both genotypes: Papss2, Cyp2b10, Gstt3, Abcc3, Nr1i3/CAR, Sult1d1,  
14  
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26 *Cyp2c29* and *Alas1*. Strikingly, CAR itself and several downstream targets of CAR were found  
27 in this list. This raised the possibility that DEHP could recruit the CAR signalling pathway, *in*  
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### 3.2. Modulation of CAR target transcripts following DEHP exposure *in vivo*

To further strengthen a hypothetical activation of CAR following DEHP exposure, we confirmed key findings of the initial gene profiling study by QPCR. Cytochrome P450 4a14 (*Cyp4a14*) [50] and vanin1 (*Vnn1*) [51] are two PPAR $\alpha$  target genes which were expected to respond to the treatment. Both genes exhibited an altered constitutive expression in PPAR $\alpha$ -deficient mice (Fig.2) and were substantially up-regulated by DEHP in wild-type mice only (*Cyp4a14*: 4.8- and 29.7-fold at 20 and 200 mg/kg/day DEHP, respectively, *Vnn1*: 5.2-fold at 200 mg/kg/day DEHP), even at the lowest dose (*Cyp4a14*).

Then we analyzed four transcripts which have been previously reported to undergo, to various levels, the influence of CAR. *Cyp2b10* transcript was markedly up-regulated by DEHP in wild-type mice (6.6 fold-change, 200 mg/kg/day), and was concomitantly modulated, but to a lower extent, in PPAR $\alpha$ -null mice (2.8 fold-increase) (Fig.3A). For *Cyp2c29*, *Cyp3a11* and *Alas1*, first, we noticed significantly lower constitutive expression levels in PPAR $\alpha$ -null mice compared to wild-type mice (2.0, 2.7 and 2.6-fold decrease respectively) (Fig.3B-D). *Cyp2c29*

1 transcript responded significantly to both doses of DEHP in wild-type mice and responded to the  
2  
3 same extent to the higher dose of DEHP even in the PPAR $\alpha$ -null mice (1.6 fold-changes in both  
4  
5 genotypes) (Fig.3B). Cyp3a11 displayed a modest, but significant, increase in wild-type mice  
6  
7 only (x 1.5, 200 mg/kg/day DEHP) (Fig.3C). Alas1 was up-regulated by DEHP, in both  
8  
9 genotypes, in comparable proportions (Fig.3D).

10  
11  
12 When we submitted the 88 genes in Table 2 to Ingenuity Pathways Analysis (IPA), the  
13  
14 CAR pathway was found to be significantly over-represented compared to the macroarray  
15  
16 composition. Out of the 21 genes which undergo the influence of CAR, according to Ingenuity  
17  
18 Knowledge base (Fig.3E), 13 were spotted on our macroarray (genes in grey in Fig.3E). 9 genes  
19  
20 out of these 13 (asterisks in Fig.3E), participated to PC2. This further supported the existence of a  
21  
22 PPAR $\alpha$ -independent response to DEHP.  
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26  
27 Based on these initial results which strongly suggested the activation of the CAR  
28  
29 signalling pathway by phthalates, we initiated a set of experiments to establish this proposal.  
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### 32 **3.3. Establishment of hepatocyte cell lines expressing a functional CAR**

33

34 As a prerequisite we had to develop a stable cell line expressing the functional CAR. An  
35  
36 initial screening conducted on readily available hepatic cell lines, revealed that the JWZ (MuSH)  
37  
38 [46] and the Hepa-I lines expressed standard levels of Retinoid X Receptor (RXR), the  
39  
40 heterodimeric partner of CAR, while CAR transcript was barely or not detectable (QPCR  
41  
42 experiments, data not shown). Using the pCR3-mCAR expression vector, we established Hepa-I-  
43  
44 CAR and JWZ-CAR cell lines which stably expressed the murine CAR nuclear receptor. Based  
45  
46 on further functional test results (not shown), we selected the JWZ-CAR line for this study. In  
47  
48 this line, the expression of the CAR transcript was 60-fold higher than in the parental JWZ cells  
49  
50 (Fig.4A). Consequently, this resulted in a 10-fold constitutive increase in Cyp2b10 mRNA  
51  
52 expression, a known downstream target of CAR (Fig.4A). This suggested a spontaneous, and at  
53  
54 least partly, nuclear localization of the active CAR protein in our JWZ-CAR line; a pattern  
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1 previously reported in HepG2 cells [44]. Indeed, we confirmed this assumption by expressing a  
2  
3 CAR-green fluorescent protein (GFP) fusion protein. The transfection of native JWZ cells with a  
4  
5 plasmid expressing GFP alone (pLVTHM) led to a conventional cytoplasmic localization of the  
6  
7 fluorescence (Fig.4B, upper lane pictures), while the same experiment with the CAR-GFP fusion  
8  
9 protein revealed a fluorescence which was restricted to the nucleus (Fig.4B, lower lane pictures).  
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11 Thus, in our JWZ-CAR model, CAR is localized in the nucleus and its constitutive activity  
12  
13 increases the expression of its prototypic target gene Cyp2b10.  
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17 For further functional investigations, we developed a panel of JWZ-CAR sub lines stably  
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19 expressing shRNAs targeting CAR. Out of the five shRNA-carrying lentiviral vectors tested, two  
20  
21 constructs displayed a convincing efficacy to lower CAR expression. We measured a reduction of  
22  
23 63% and 69% of CAR mRNA level with pLKO-SH1012 and pLKO-SH767, respectively  
24  
25 (Fig.4C). This translated into the parallel reduction of the constitutive level of the Cyp2b10  
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27 mRNA by 69% and 70% (Fig.4C).  
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### 31 **3.4. DEHP regulates gene expression through the CAR signalling pathway**

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34 Firstly, we evaluated the response of the CAR pathway to an agonist (TCPOBOP, [52]) or  
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36 an inverse agonist (androstenol, [53]) in the JWZ-CAR recombinant cells. TCPOBOP doubled  
37  
38 the level of Cyp2b10. Androstenol, which alone reduced Cyp2b10 constitutive expression,  
39  
40 abolished Cyp2b10 induction by TCPOBOP (Fig.5A, columns 1-4).  
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43 The *in vivo* effect of DEHP on Cyp2b10 hepatic transcript was reproduced (Fig.5A) in the  
44  
45 recombinant JWZ-CAR cell line. Indeed, the incubation of the cells with 100 $\mu$ M of DEHP  
46  
47 resulted in a two-fold increase of Cyp2b10 mRNA, equivalent to that observed with TCPOBOP  
48  
49 (Fig.5A, column 5). Similarly, androstenol abolished the induction of Cyp2b10 by DEHP, thus  
50  
51 supporting the involvement of CAR in this regulation (Fig.5A, column 6). In our JWZ-CAR cell  
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53 line MEHP (100 $\mu$ M), the main DEHP metabolite, was unable to increase Cyp2b10 mRNA  
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55 (Fig.5B).  
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1 Finally, using the SH1012 and SH767 cell lines expressing CAR-targeting shRNAs, we  
2 showed that a ~60% reduction in CAR expression led to a loss of the ability to modulate  
3 Cyp2b10 mRNA level by TCPOBOP, androstenol, or DEHP (Fig.5C). Thus, this established that,  
4 like TCPOBOP, DEHP regulates the prototypic Cyp2b10 target by activating CAR.  
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### 10 **3.5. DEHP regulates CYP2B6 expression in human primary hepatocytes**

11 To evaluate whether the activation of CAR by DEHP could be extrapolated to humans,  
12 we tested the effects of DEHP on CYP2B6 expression in two independent cultures of human  
13 primary hepatocytes. Consistently, DEHP dose-dependently increased the expression of CYP2B6  
14 at 50 and 100 $\mu$ M of DEHP in both primary hepatocyte cultures (Fig.6).  
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## 25 **4. Discussion**

26 Human exposure to phthalates inevitably occurs due to their use in plastics and consumer  
27 products [2]. In the adult, the main concerns associated to phthalate exposure are linked to  
28 hepatocarcinogenesis [10], endocrine [9, 25] and metabolic disruption [27]. Despite many  
29 studies, the mechanisms involved in these effects remain poorly understood.  
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36 In this study, we confirm the responsiveness of PPAR $\alpha$  pathway to DEHP (activation  
37 even at the 20 mg/kg/day low dose, Fig.2A). And when abrogating the dominant signal elicited  
38 by PPAR $\alpha$  using PPAR $\alpha$ -null mice, we revealed the underlying activation of the hepatic CAR  
39 pathway (Table 2, Fig.1 and 3). The activation of CAR by DEHP was then demonstrated using a  
40 CAR-expressing cell line which was exposed either to a CAR inverse agonist (androstenol,  
41 Fig.5A) or to shRNAs targeting CAR (Fig.5C).  
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51 Consistent with our finding, it was recently reported that DEHP may activate Cyp2b10  
52 promoter, but this was done only with a gene reporter system [54]. Our study is the first report on  
53 DEHP activation of Cyp2b10 gene both *in vitro* and *in vivo*. It also provides the first *in vitro*  
54 demonstration that CAR is involved in the regulation of Cyp2b10 by DEHP, which could  
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1 otherwise be attributed to PXR. CAR activation by phthalates may not be restricted to mice  
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3 species and to DEHP since an other phthalate, the Di-n-butyl phthalate (500mg/kg/day) was  
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5 shown to increase the CAR-target Cyp2b1 transcript in late rat embryos (E:19) [55].  
6  
7 Interestingly, another group has very recently reported that the CAR2 splice variant of human  
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9 CAR is activated by DEHP [56]. Similar to their observations using in vitro reporter assays [56],  
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11 we show here that MEHP, the main DEHP metabolite does not induce Cyp2b10 in our JWZ-  
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13 CAR cell line. It remains to be established whether DEHP or one of its metabolite other than  
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15 MEHP directly binds to mouse and/or human CAR.  
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20 CAR (Nr1i3) was first described as a xenobiotic-sensing nuclear receptor that regulates  
21  
22 the expression of hepatic enzymes and transporters involved in xenobiotic metabolism [57]. It is  
23  
24 closely related to PXR (Nr1i2) and these receptors share both ligands [58] and target genes [59].  
25  
26 Indeed, DEHP and MEHP also activate mouse and human PXR [34, 60] which result in CYP3A  
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28 induction. *In vivo*, we observed a higher induction by DEHP of Cyp2b10 expression (Fig. 3A)  
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30 compared to Cyp3a11 (Fig.3C), suggesting that the CAR pathway is at least as responsive as the  
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32 PXR pathway to DEHP.  
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36 Importantly, the xenobiotic metabolizing systems induced by CAR and PXR are also  
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38 involved in the metabolism of endogenous molecules including steroid and thyroid hormones.  
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40 Both receptors protect the body from the deleterious accumulation of bilirubin [61, 62] and bile  
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42 acids [63] through hepatic induction of several enzyme genes. Additionally, many CAR and PXR  
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44 target genes, including CYP3A and CYP2B, are involved in steroid hormone metabolism and  
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46 their induction could contribute to the endocrine disruption activities of drugs and contaminants  
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48 [64, 65]. CYP2B6 induction following DEHP exposure of human hepatocytes confirmed a  
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50 possible extrapolation to humans of this DEHP-mediated CAR activation observed in mice  
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52 (Fig.6, [56]). This induction could contribute to increased hepatic testosterone metabolism,  
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1 leading to a reduction of circulating testosterone levels as observed in workers exposed to high  
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3 levels of phthalates [9].  
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6 Similarly, the CAR-dependent induction of genes involved in hepatic phase II xenobiotic  
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8 metabolism is responsible for increased thyroid hormone metabolism [66]. Consistently, CAR-  
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10 deficient mice loose more weight than wild-type controls during caloric restriction, likely due to a  
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12 higher metabolic rate driven by higher thyroid hormone levels [67]. Interestingly, recent studies  
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14 have suggested an inverse association between phthalate exposure and thyroid hormone levels in  
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16 men [68] and pregnant women [69]. Given our observations, it is conceivable that CAR  
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18 activation by DEHP contributes to a reduction in thyroid hormones which could favor a positive  
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20 energy balance and weight gain in a context of high caloric intake. Accordingly, we have  
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22 observed that high DEHP exposure (1100 mg/Kg/day for 2 weeks) led to a significant reduction  
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24 in plasma total T4 (data not shown). CAR and PXR are also increasingly recognized as important  
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26 regulators of genes involved in lipid and glucose homeostasis (for review: [70, 71]. In particular,  
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28 CAR activation reduces the expression of critical genes involved in fatty acid oxidation [72], bile  
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30 acid synthesis and gluconeogenesis [73]. Further studies should evaluate the potential role of  
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32 these regulations in mediating metabolic disruption by phthalates in light of their potential to  
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34 activate both PXR and CAR.  
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41 Finally, CAR also mediates the hepatocarcinogenic effects of its xenobiotic ligands [74],  
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43 suggesting that it may contribute to PPAR $\alpha$ -independent hepatocarcinogenesis [13] following  
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45 high dose DEHP exposure. Indeed, we have observed that high DEHP exposure (1100  
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47 mg/kg/day, 14 days) in mice induces the hepatic expression of Mdm2 (2.1-fold, data not shown),  
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49 a direct CAR target gene that contributes to hepatocyte proliferation [74].  
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53 Overall, our results demonstrate that DEHP activates the CAR pathway which results in  
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55 the induction of several hepatic target genes *in vivo*. This adds DEHP to the growing list of  
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57 environmental contaminants activating CAR [54, 75-77], further stressing the need to evaluate  
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1 the specific role of this NR in mediating the array of deleterious carcinogenic, endocrine and  
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3 metabolic effects associated with exposure to these compounds and their mixtures.  
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## Figure Legends

Figure 1. Principal Component Analysis (PCA) of liver transcriptional signature. PCA was performed on a data set containing all 60 samples. 88 transcripts were initially analyzed for each sample. (A) Individual projection in PC1 and PC2 (first principal plan) is shown for liver of wild-type (square) and PPAR $\alpha$ <sup>-/-</sup> mice (circle) according to treatments of 20 (light grey), and 200 (black) mg of DEHP /kg/day or vehicle (white). *n* = 10 animals in each treated or control group. (B) Correlation circle of gene expression variables. Genes with the highest correlation to the first plan are represented by vectors (corresponding names along dotted lines).

Figure 2. Effect of DEHP administration on hepatic mRNA levels of PPAR $\alpha$  dependent genes. Relative expressions of Cyp4a14 (A) and Vanin1 (B) mRNA were determined using Q-PCR normalized to the expression of TBP. Values shown are the mean  $\pm$  S.E.M. (*n* = 10 for each group). Significant differences were analyzed by one-way ANOVA followed by Student's *t* test. An asterisk (\*) denotes a significant difference compared with control condition (vehicle alone) while a significant difference between the two genotypes was represented with "#". *P* < 0.05.

Figure 3. Effect of DEHP administration on hepatic mRNA levels of CAR target genes. Relative expressions of Cyp2b10 (A), Cyp2c29 (B), Cyp3a11 (C) and Alas1 (D) mRNA were determined using Q-PCR normalized to the expression of TBP. Values shown are the mean  $\pm$  S.E.M. (*n* = 10 for each group). Significant differences were analyzed by one-way ANOVA followed by Student's *t* test. An asterisk (\*) denotes a significant difference compared with control condition (vehicle alone) while a significant difference between the two genotypes was represented with "#". *P* < 0.05. (E) Network representation of the genes identified by Ingenuity Pathway Analysis (IPA) in constitutive androstane receptor pathway. Grey indicates regulated genes in our study with treatment and/or genotype effect. Genes associated to the second axis of PCA (Fig. 1) were shown with an asterisk (\*).

Figure 4. New murine hepatocyte cell line expressing the CAR nuclear receptor and CAR directed shRNA. (A) Q-PCR results of relative expressions of CAR and Cyp2b10 in the parental JWZ cell line and the newly established JWZCAR cell line. Values shown are the mean  $\pm$  S.E.M. (*n* = 3 for each cell line). (B) Analysis by confocal microscopy of subcellular localization of CAR protein in JWZ hepatocyte cell line. JWZ cells were transfected either with the pLVTHM vector expressing a native EGFP protein or the pEGFP-CAR construct expressing a EGFP-tagged CAR protein. (C) CAR and Cyp2b10 mRNA knockdown following infection of JWZCAR cell line with the lentiviral vectors pLKO-SH1012 or pLKO-SH767.

Figure 5. CAR dependent effect of DEHP and MEHP on Cyp2b10 mRNA levels. Cells were incubated for 48 hours in the presence of DMSO, TCPOBOP (200 nM), androsthenol (5  $\mu$ M), DEHP (100 $\mu$ M), MEHP (100 $\mu$ M) or a mix of TCPOBOP and androsthenol or DEHP and androsthenol. Cyp2b10 mRNA were then quantified by QPCR. Values shown are the mean  $\pm$  S.E.M. (*n* = 3 for each cell treatment).

(A) Cyp2b10 mRNA modulation after TCPOBOP, androsthenol or DEHP treatment in the JWZCAR cell line. Significant differences were analyzed by one-way ANOVA followed by Student's *t* test. An asterisk (\*) denotes a significant difference compared with control condition (vehicle alone), *P* < 0.05. (B) MEHP (100 $\mu$ M) does not induce Cyp2b10 expression in the JWZCAR cell line. (C) No more significant Cyp2b10 mRNA modulation after treatment with TCPOBOP, androsthenol or DEHP in the CAR-knockdown cell lines (SH1012 and SH767).

1 Figure 6. Effect of DEHP on CYP2B6 mRNA level in human primary hepatocytes. Q-PCR  
2 results of relative expressions of CYP2B6 in primary hepatocytes derived from 2 patients  
3 (patient1 and patient 2) treated with 2 concentrations of DEHP (50 $\mu$ M and 100 $\mu$ M).  
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**Table 1:** Oligonucleotides sequence for real time PCR

Gene	NCBI Refseq	Forward primer (5'-3')	Reverse primer (5'-3')
Alas1	NM_020559	CAAAGAAACCCCTCCAGCC	GCTGTGTGCCGTCTGGAGT
CAR	NM_009803	GCTGCAAGGGCTTCTTCAGA	CCTTCCAGCAAACGGACAGA
Cyp2b10	NM_009999	TTTCTGCCCTTCTCAACAGGAA	ATGGACGTGAAGAAAAGGAACAAC
Cyp2c29	NM_007815	GCTCAAAGCCTACTGTCA	CATGAGTGTAATCGTCTCA
Cyp3a11	NM_007818	TCACACACACAGTTGTAGGCAGAA	GTTTACGAGTCCCATATCGGTAGAG
Cyp4a14	NM_007822	TCAGTCTATTCTGGTGCTGTTC	GAGCTCCTTGTCTTCAGATGGT
Tbp	NM_013684	ACTTCGTGCAAGAAATGCTGAA	GCAGTTGTCCGTGGCTCTCT
Vnn1	NM_011704	ATGAGGTTTATGCCTTTGGAGC	CCACAGGTGCGTAAATTGGTAG
CYP2B6	NM_000767	GGCCATACGGGAGGCCCTTG	AGGGCCCCCTTGGATTCCG
GAPDH	NM_002046	GGTCGGAGTCAACGGATTGGTCG	CAAAGTTGTCATGGATGACC

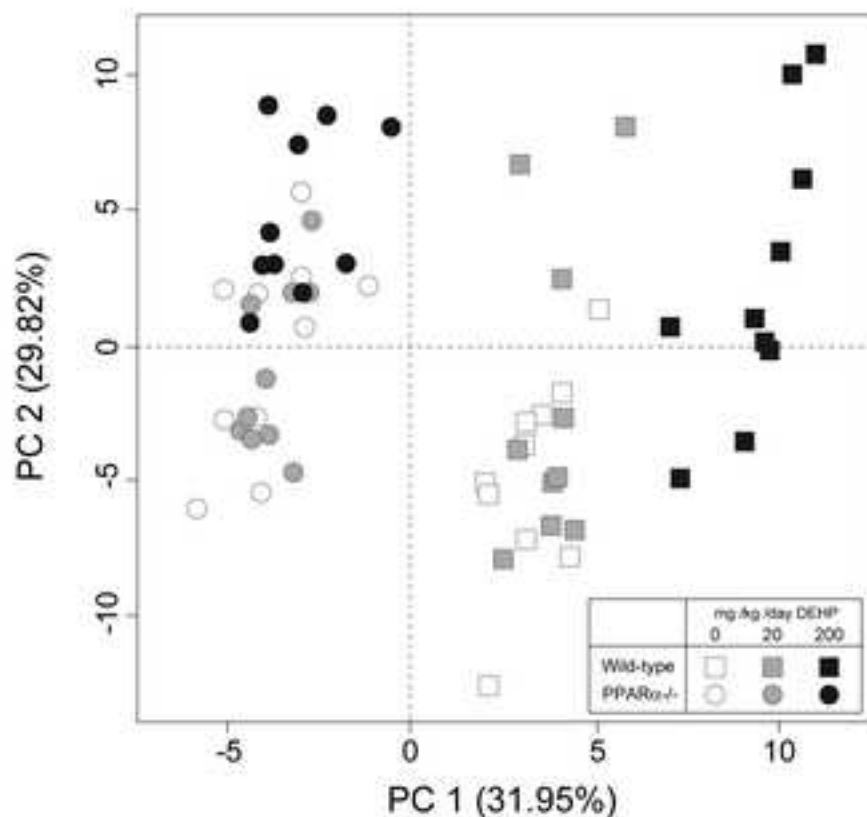
Table 2: Liver gene expression modulations induced by DEHP treatment

Gene description		Fold change			
Function & gene name	GenBankTM RefSeq	Symbol	PPAR $\alpha$ -/- 200 / ctrl	Wild-type 200 / ctrl	Untreated PPAR $\alpha$ -/- / wt
<b>fatty acid metabolism</b>					
acetyl-Coenzyme A acyltransferase 1A	NM_130884	Acaa1a		2.9	
acetyl-Coenzyme A dehydrogenase, medium chain	NM_007382	Acadm		1.4	
acyl-CoA thioesterase 1	NM_012006	Acot1		3.8	
acyl-Coenzyme A oxidase 1, palmitoyl	NM_015729	Acox1	1.3	2.7	-1.5
cytochrome P450, family 2, subfamily a, polypeptide 5	NM_007812	Cyp2a5	1.6		-1.8
cytochrome P450, family 4, subfamily a, polypeptide 10	NM_010011	Cyp4a10		5.4	-3.4
cytochrome P450, family 4, subfamily a, polypeptide 12	NM_177406	Cyp4a12		3.0	1.5
cytochrome P450, family 4, subfamily a, polypeptide 14	NM_007822	Cyp4a14		7.6	-1.7
dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenzyme A isomerase)	NM_010023	Dci		1.7	-1.3
enoyl coenzyme A hydratase 1, peroxisomal	NM_016772	Ech1		1.8	-2.9
enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	NM_023737	Ehhadh		9.3	
hydroxyacyl-Coenzyme A dehydrogenase(trifunctional protein), alpha subunit	NM_178878	Hadha		1.4	
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	NM_008256	Hmgcs2		1.3	-1.9
peroxisomal delta3, delta2-enoyl-Coenzyme A isomerase	NM_011868	Peci		1.8	-1.8
peroxisomal biogenesis factor 11a	NM_011068	Pex11a		1.5	
solute carrier family 27 (fatty acid transporter), member 5	NM_009512	Slc27a5	1.3		
<b>fatty acid transport</b>					
ATP-binding cassette, sub-family D (ALD), member 3	NM_008991	Abcd3		1.3	
CD36 antigen / fatty acid translocase	NM_007643	Cd36		1.5	
carnitine palmitoyltransferase 2	NM_009949	Cpt2		1.4	-1.6
diazepam binding inhibitor	NM_007830	Dbi		1.6	
fatty acid binding protein 1, liver	NM_017399	Fabp1		2.0	
fatty acid binding protein 2, intestinal	NM_007980	Fabp2		1.7	
<b>lipoprotein metabolism</b>					
apolipoprotein A-IV	NM_007468	Apoa4		-1.8	8.4
apolipoprotein A-V	NM_080434	Apoa5			-1.4
apolipoprotein C-III	NM_023114	Apoc3			1.4
phospholipid transfer protein	NM_011125	Pltp		1.4	
scavenger receptor class B, member 1	NM_016741	Scarb1			1.4
very low density lipoprotein receptor	NM_013703	Vldlr		1.4	-1.6
<b>fatty acid synthesis</b>					
elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	NM_007703	Elovl3			-2.0
fatty acid desaturase 1	NM_146094	Fads1			-1.4
fatty acid desaturase 2	NM_019699	Fads2			-1.6
lipin 1	NM_015763	Lpin1		-1.6	
stearoyl-Coenzyme A desaturase, delta 9 desaturase	NM_009127	Scd1		1.6	-4.3
thyroid hormone responsive SPOT14 homolog (Rattus)	NM_009381	Thrsp	1.7		1.5
<b>cholesterol synthesis</b>					
cytochrome P450, family 7, subfamily b, polypeptide 1	NM_007825	Cyp7b1			2.1
farnesyl diphosphate farnesyl transferase 1	NM_010191	Fdft1		1.5	1.5
farnesyl diphosphate synthetase	NM_134469	Fdps		1.7	1.6
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	NM_145942	Hmgcs1		1.4	1.4
hydroxysteroid 11-beta dehydrogenase 1	NM_008288	Hsd11b1	1.3		1.4
hydroxysteroid (17-beta) dehydrogenase 4	NM_008292	Hsd17b4		1.7	
<b>xenobiotic metabolism</b>					
ATP-binding cassette, sub-family C (CFTR/MRP), member 2	NM_013806	Abcc2		1.4	
ATP-binding cassette, sub-family C (CFTR/MRP), member 3	NM_029600	Abcc3		1.3	
aldehyde dehydrogenase family 1, subfamily A1	NM_013467	Aldh1a1	1.6	1.4	
aldehyde dehydrogenase family 3, subfamily A2	NM_007437	Aldh3a2		1.4	
cytochrome P450, family 1, subfamily a, polypeptide 1	NM_009992	Cyp1a1		1.5	1.7
cytochrome P450, family 2, subfamily b, polypeptide 10	NM_009998	Cyp2b10		1.4	
cytochrome P450, family 2, subfamily c, polypeptide 29	NM_007815	Cyp2c29	1.7	1.4	1.5
cytochrome P450, family 2, subfamily c, polypeptide 38	NM_010002	Cyp2c38			1.4
cytochrome P450, family 2, subfamily j, polypeptide 5	NM_010007	Cyp2j5		1.3	
cytochrome P450, family 3, subfamily a, polypeptide 11	NM_007818	Cyp3a11	2.0		-2.0
glutathione S-transferase, mu 1	NM_010358	Gstm1		1.3	
glutathione S-transferase, theta 3	NM_133994	Gstt3	1.4		
solute carrier family 10 (sodium/bile acid cotransporter family), member 1	NM_011387	Slc10a1			1.4
solute carrier organic anion transporter family, member 1a1	NM_013797	Slco1a1			1.4
solute carrier organic anion transporter family, member 1a4	NM_030687	Slco1a4			2.0
sulfoltransferase family 1D, member 1	NM_016771	Sult1d1			1.4
UDP glucuronosyltransferase 1 family, polypeptide A1	NM_201645	Ugt1a1	1.4		
UDP glucuronosyltransferase 1 family, polypeptide A9	NM_201644	Ugt1a9	1.3		
<b>others</b>					
aminolevulinic acid synthase 1	NM_020559	Alas1	2.7	2.1	
actin, beta, cytoplasmic	NM_007393	Actb		1.4	
angiotensin-like 4	NM_020581	Angptl4		1.4	-2.0
B-cell leukemia/lymphoma 3	NM_033601	Bcl3		1.4	
cyclin-dependent kinase inhibitor 1A (P21)	NM_007669	Cdkn1a		1.4	-1.6
glutamate oxaloacetate transaminase 1, soluble	NM_010324	Got1	1.5		
gulonolactone (L-) oxidase	NM_178747	Gulo			1.4
inhibitor of DNA binding 2	NM_010496	Id2	1.4		
inhibitor of kappaB kinase gamma	NM_010547	Ikbkg			2.2
lymphoid enhancer binding factor 1	NM_010703	Lef1		1.8	
lipin 2	NM_022882	Lpin2		1.4	-1.4
5,10-methylenetetrahydrofolate reductase	NM_010840	Mthfr			2.2
5-methyltetrahydrofolate-homocysteine methyltransferase	XM_910457	Mtr			1.4
major urinary protein 2	NM_008647	Mup2			2.5
3'-phosphoadenosine 5'-phosphosulfate synthase 2	NM_011864	Papss2			1.3
proliferating cell nuclear antigen	NM_011045	Pcna		1.3	
pyruvate dehydrogenase kinase, isoenzyme 4	NM_013743	Pdk4		3.4	
paraoxonase 1	NM_011134	Pon1			1.3
peroxisome proliferative activated receptor, gamma, coactivator 1 beta	NM_133249	Ppargc1b		1.5	1.5
transformation related protein 53	NM_011640	Trp53			1.3
uncoupling protein 3 (mitochondrial, proton carrier)	NM_009464	Ucp3			-1.6
<b>nuclear receptor</b>					
nuclear receptor subfamily 1, group H, member 2	NM_009473	Nr1h2		1.3	
nuclear receptor subfamily 1, group I, member 3	NM_009803	Nr1i3			1.4
nuclear receptor subfamily 4, group A, member 1	NM_010444	Nr4a1			1.3
peroxisome proliferator activated receptor alpha	NM_011144	Ppara		1.3	
retinoic acid receptor, beta	NM_011243	Rarb			1.4
retinoic acid receptor, gamma	NM_011244	Rarg			1.6
thyroid hormone receptor alpha	NM_178060	Thra		1.4	-1.4
thyroid hormone receptor beta	NM_009380	Thrb			-1.3
vitamin D receptor	NM_009504	Vdr			-1.5

The table reports the fold change of each DEHP-regulated transcript which has been recorded, in the liver, from macroarray analysis using INRArray 01.4 gene set. Significant differences were analyzed by one-way ANOVA followed by Student's *t* test. Resulting *p*-values were adjusted by the Benjamini-Hochberg procedure to control the FDR at 10%. Genes with adjusted *P*<0.10 and with at least a 1.3-fold change in expression (induction or repression) are presented.

Figure 1.

A.



B.

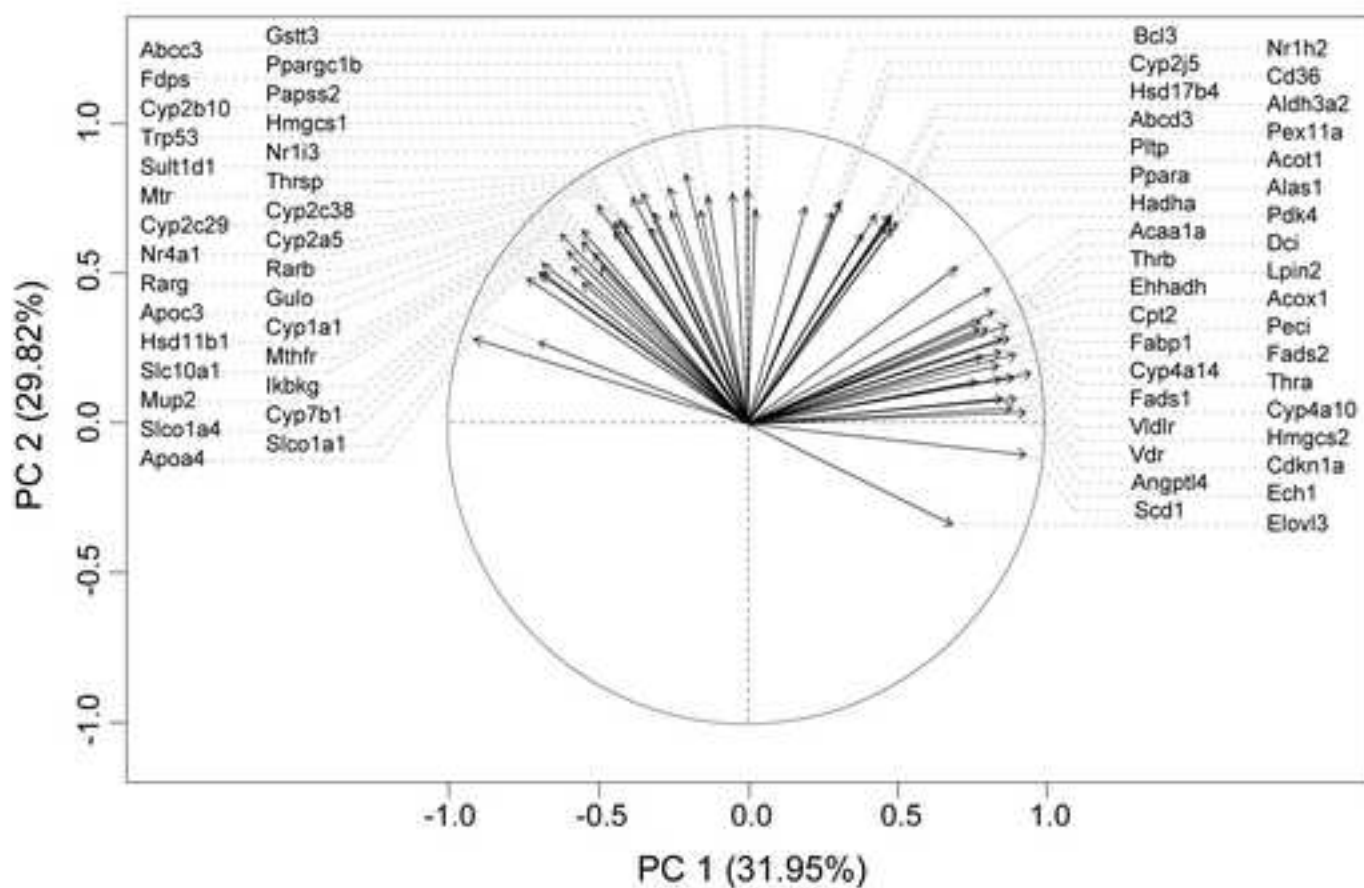


Figure 2.

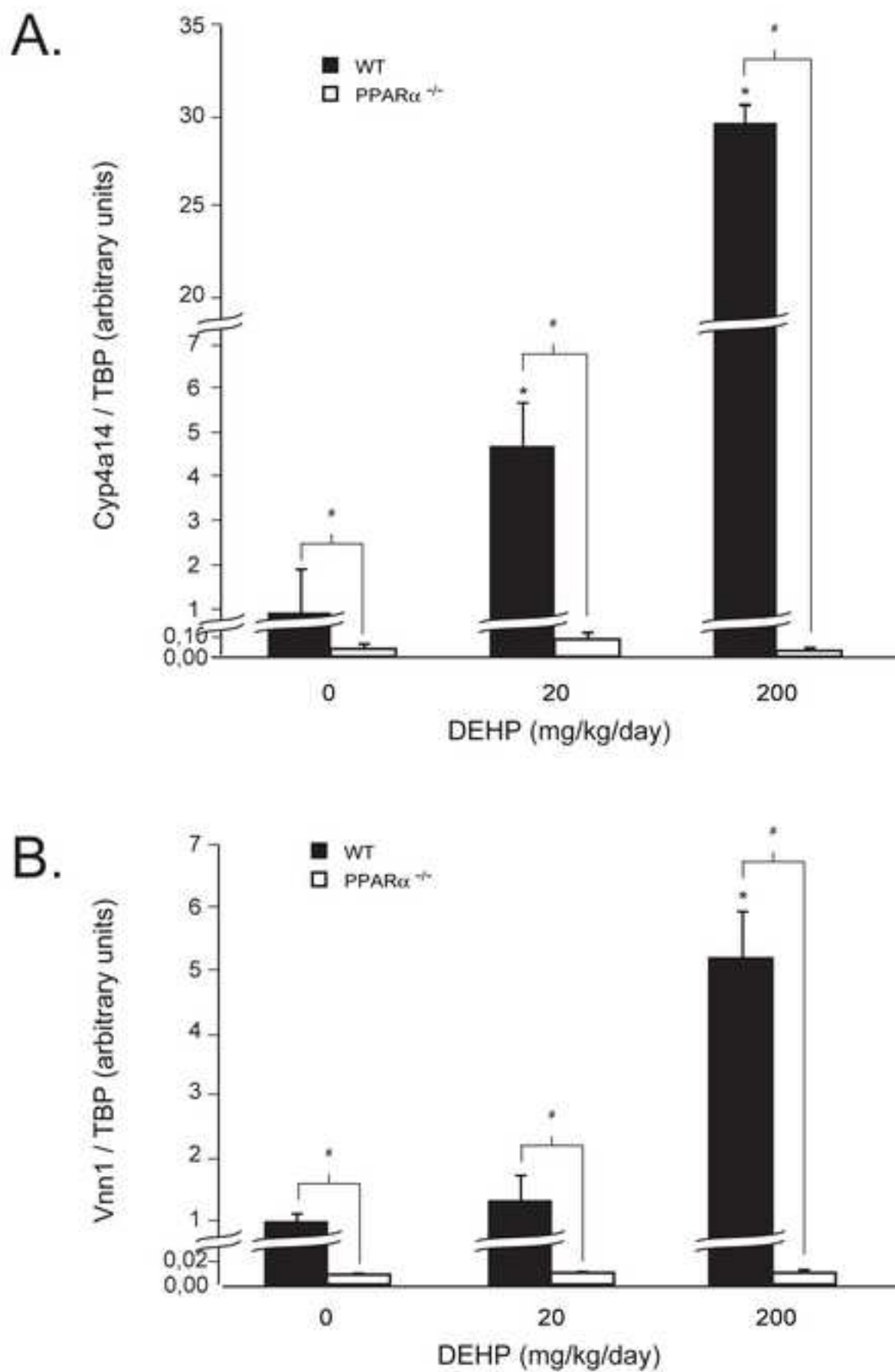


Figure 3.

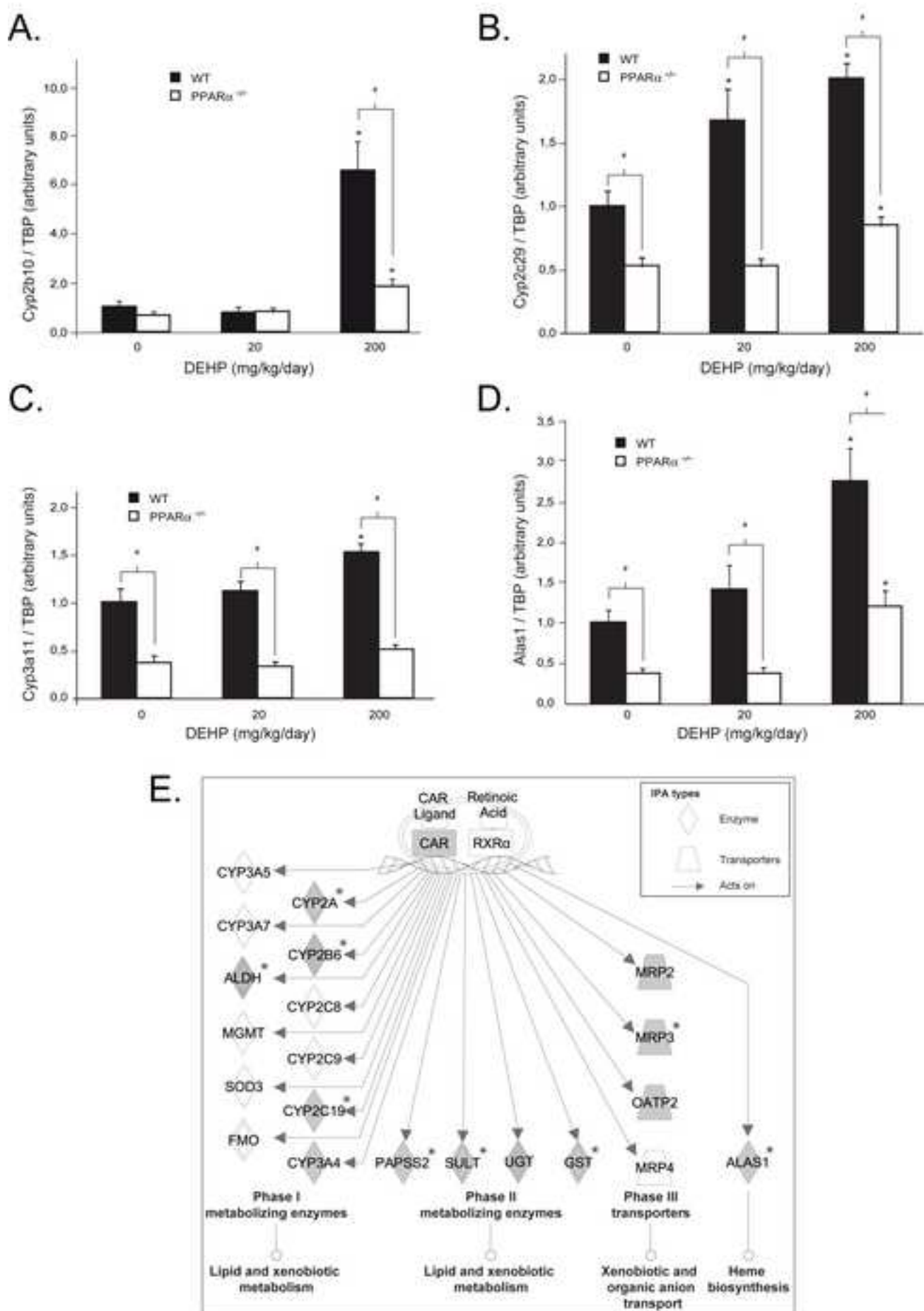


Figure 4.

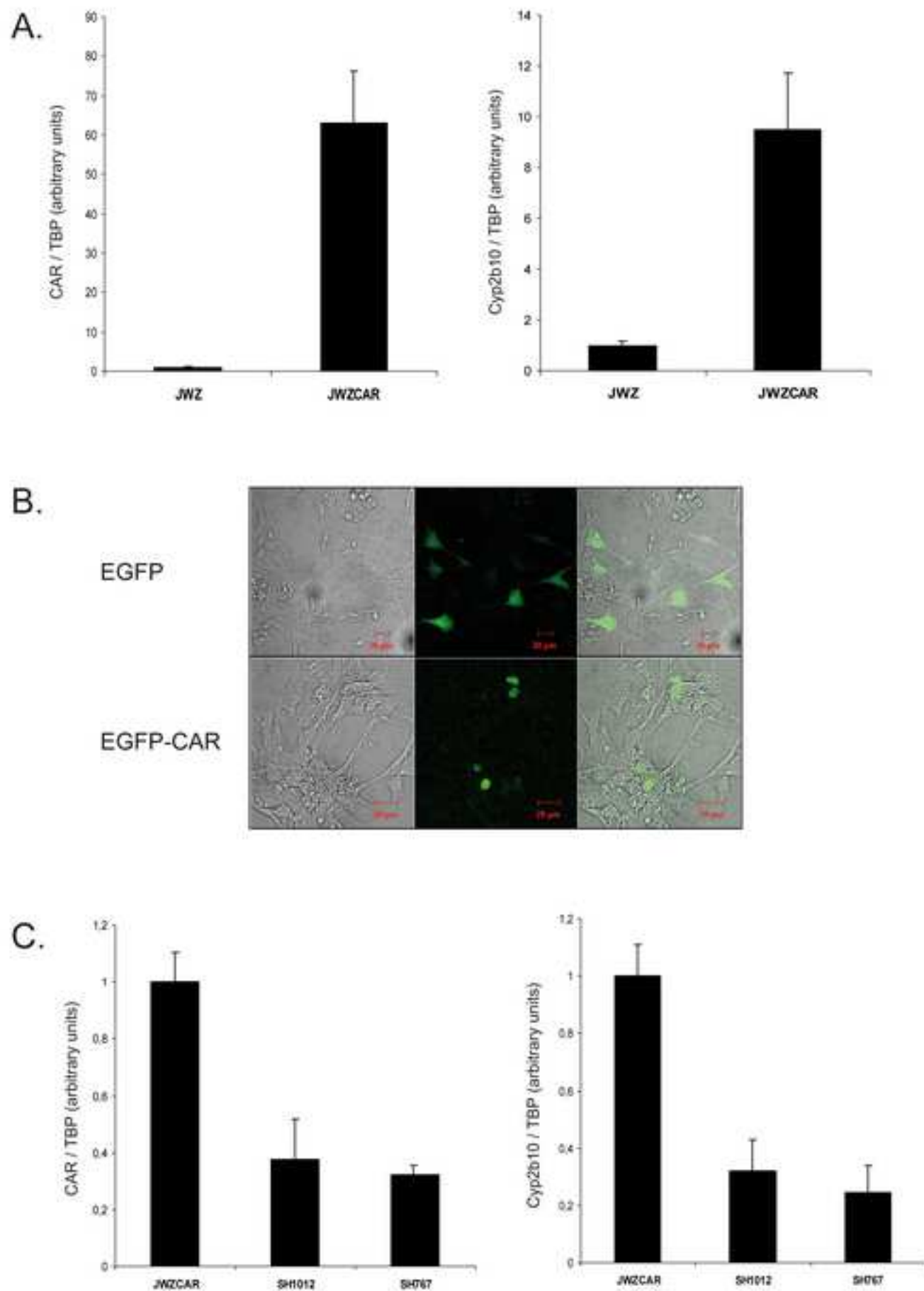


Figure 5.

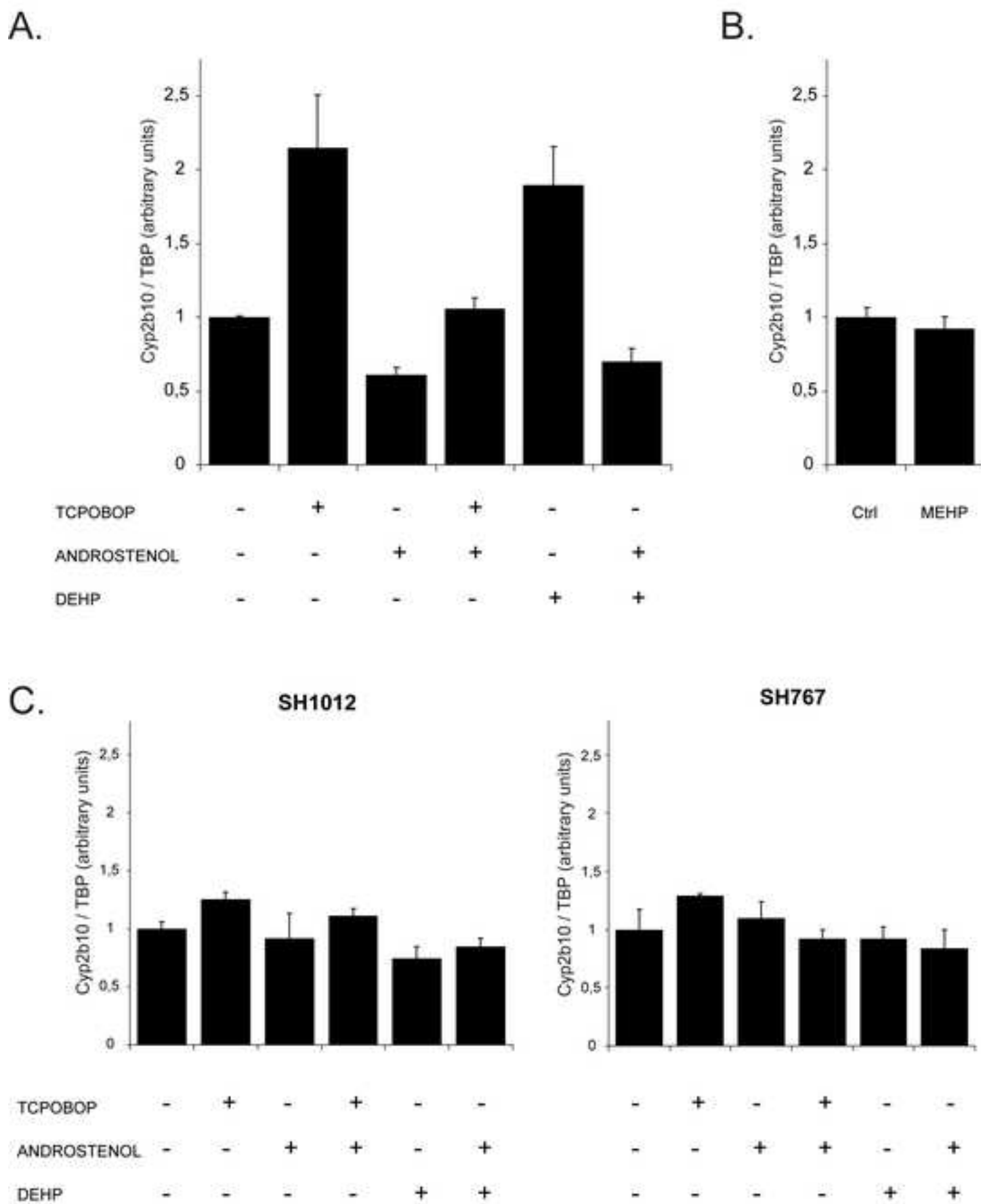
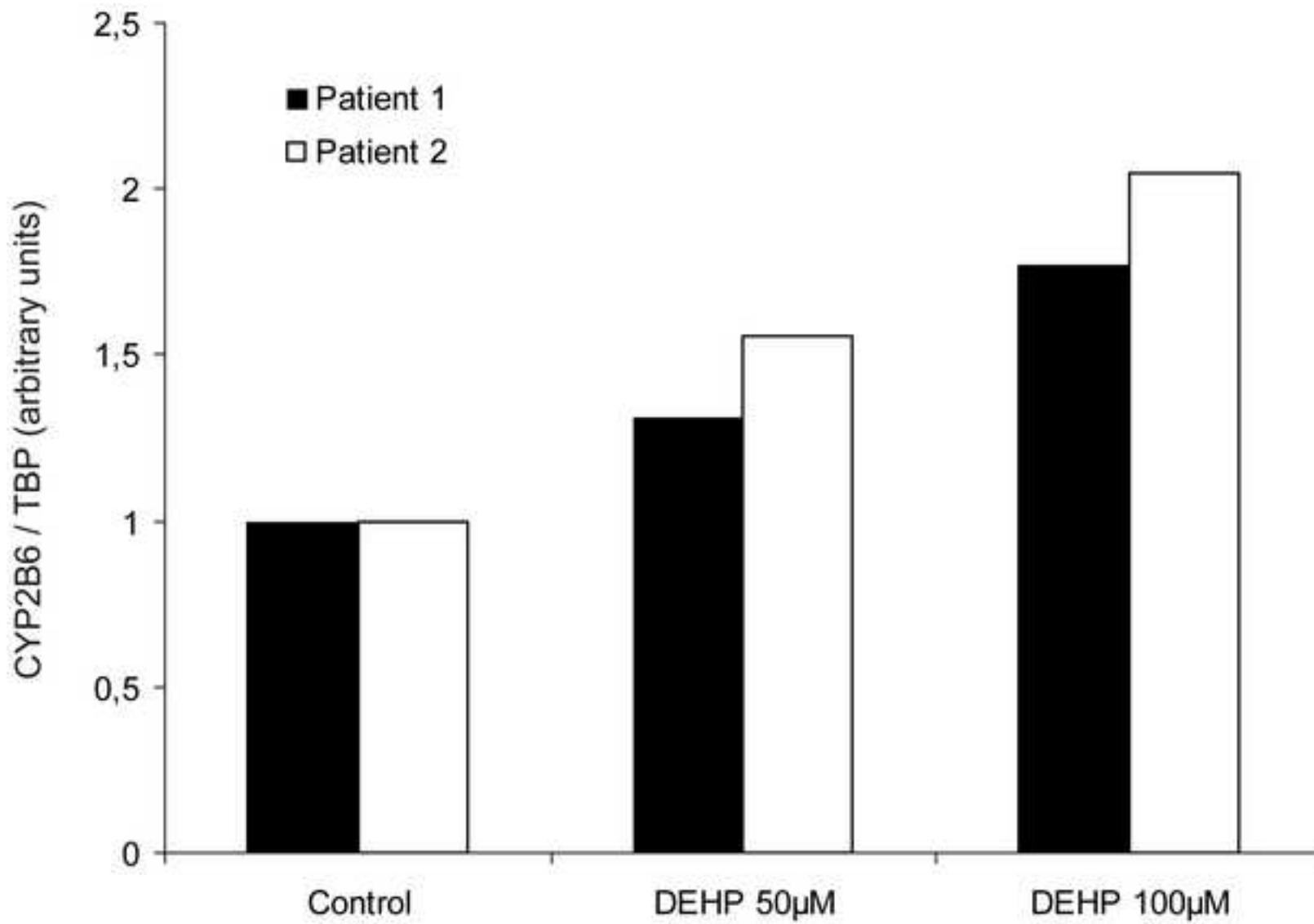
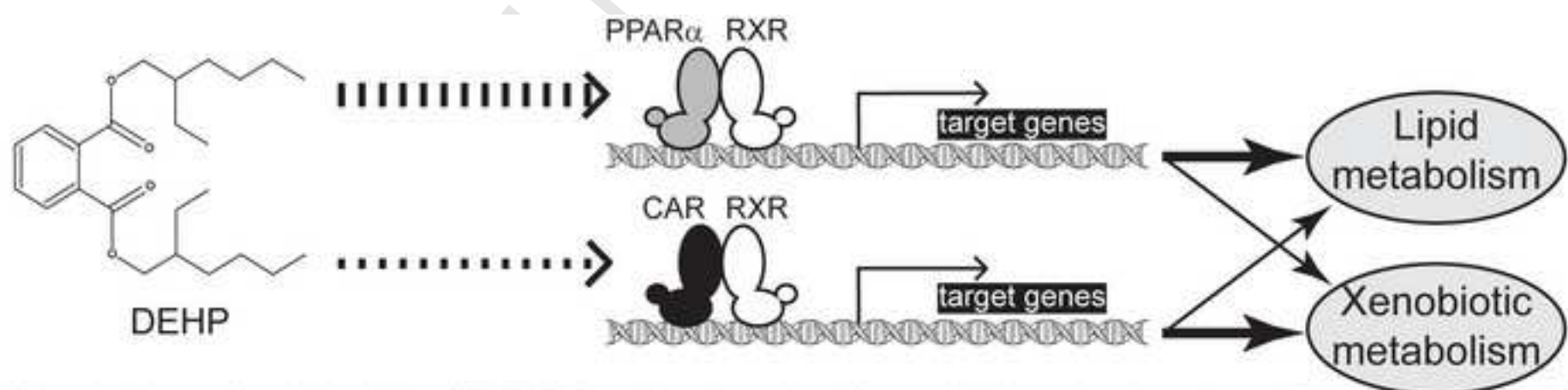


Figure 6.







Di-(2-ethylhexyl)-phthalate (DEHP) activates the Constitutive Androstane Receptor (CAR): a novel PPAR $\alpha$  independent pathway sensitive to phthalates.