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Di-(2-ethylhexyl)-phthalate (DEHP) activates the constitutive androstane 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 (PPARα) 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 PPARα activation, and the putative activation of additional signalling pathways. Wild-type and PPARα-deficient mice were exposed to different doses of DEHP. Gene expression profiling delineated the role of PPARα and revealed a PPARα-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 PPARα-dependent mechanisms and suggested a controversial low relevance to humans [11,12]. However, while PPARα-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

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could disturb the gonadal development in the embryo through the modification of androgens bioavailability [24]. Sustained exposure to phthalates through adulthood could also contribute to decreased testosterone levels [9] or semen quality [25].

Recently, two independent cross sectional studies of the National Health And Nutrition Examination Survey [NHANES, 1999–2002] have provided consistent conclusions which associate several urinary phthalates metabolites with key features of the metabolic syndrome (body mass index, abdominal adiposity and insulin resistance) [18,19]. Based on several studies, it has been suggested that the onset of the metabolic syndrome could be favoured by prolonged exposure to low concentrations of environmental pollutants, including phthalates, which are collectively referred to as *environmental obesogens* [18,26,27]. However, the mechanisms involved in these new aspects of phthalate metabolic impact remain poorly understood. At the molecular level, the activation of the three isoforms of the peroxisome proliferator-activated receptor (PPAR, α, β and γ) by phthalate metabolites and the resulting metabolic consequences have been extensively documented [28]. In adipose cells, mono-ethylhexyl-phthalate (MEHP), a potent metabolite of DEHP, activates PPARγ (Nr1c3) and promotes adipogenesis [29]. In liver, another metabolically relevant organ, phthalates activate PPARα (Nr1c1) and behave as prototypical peroxisome proliferators (PP) [30], with rodents being more responsive than primates [31]. Gene expression profiling studies have further confirmed the striking over representation of PP-responding genes in the liver of rodents exposed to DEHP [32,33]. Aside from PPARα-dependent effects, phthalates were shown to activate the murine and human pregnane X nuclear receptor (PXR, Nr1i2), thus increasing the transcription of cytochrome P450 3A (Cyp3A) [34,35].

The main objective of this study was to investigate the influence of DEHP on the network of hepatic nuclear receptors, while leaving aside the PPARα-dependent response, which has already been well established. Using gene expression profiles dedicated to NR pathways, we identified the constitutive androstane receptor (CAR, Nr1i3) as a putative phthalate target. Then we developed specific recombinant cellular tools to establish that phthalates activate the CAR signalling pathway. Finally, we showed that DEHP dose-dependently induce the expression of the CAR target gene CYP2B6 in primary human hepatocytes. This original finding is discussed in view of the crucial role played by the liver in controlling energy homeostasis and the recent findings which associate phthalate exposure to the onset of the metabolic syndrome in men [18,19].

2. Materials and methods

2.1. Materials

Di-(2-ethylhexyl)-phthalate (CAS No. 117-81-7, ≥97% purity), 5α-androst-16-en-3α-ol (androstenol), 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), dimethylsulfoxide (DMSO), dexamethasone, geneticin and sodium butyrate were purchased from Sigma (Lyon, France). Cell culture medium (DMEM), fetal bovine serum (FBS), glutamine, penicillin and streptomycin, trypsine, lipofectamine 2000 and sodium pyruvate were purchased from Invitrogen (Cergy Pontoise, France). Mono-(2-ethylhexyl) phthalate (MEHP) was kindly provided by Dr. Jegou (Inserm-U625, Rennes, France).

2.2. Animals and maintenance

Male PPARα-null mice on a C57BL/6J genetic background [36,37], and aged-matched wild-type counterparts (Charles River, Les Oncins, France; 4 weeks acclimatization) were housed in polycarbonate cages at 22 ± 2 °C on a 12-h light/dark cycle and allowed free access to water and food. *In vivo* studies were conducted under European Union Guidelines for the use and care of laboratory animals and were approved by an independent ethics committee.

2.3. Experimental design, body fluids and organs sampling

At the age of 18–22 weeks with an average initial body weight of 26.6 g (±1.8; S.D.), mice from both genotypes were randomly assigned into six groups ($n = 10$ per group). DEHP, in solution into corn oil, was administered for 21 days by daily gavages (corn oil control group, 20 or 200 mg/(kg day) of DEHP). Blood samples were collected at the retro-orbital sinus with heparin-coated capillaries on 3 days (−1, 10 and 21). Plasma was prepared by centrifugation at 16000 × *g*, for 10 min and kept at −80 °C. At day 21, mice were euthanized by cervical dislocation and the liver was removed, weighed, dissected and snap-frozen in liquid nitrogen and stored at −80 °C until being used for transcriptomic analysis.

2.4. Transcriptomic analyses

Organ-specific transcriptomic profiles were obtained using a nuclear receptors-dedicated cDNA macroarray (INR*Array* 01.4) previously developed and validated [38,39]; full list of 320 cDNA probes available at http://www.inra.fr/internet/Centres/toulouse/pharmacologie/pharmaco-moleculaire/technologie/inrarray-01-4.html. Total hepatic RNA was extracted with TRIzol® reagent (Invitrogen, Cergy Pontoise, France) according to the manufacturer's protocol. RNA integrity was controlled on a Bioanalyzer 2100 (Agilent, Massy, France) and assayed at 260 nm on a Lambda 650 spectrophotometer (PerkinElmer, Toulouse, France). RNA labelling and hybridization to INR*Array* 01.4 were performed as previously described [39]. The raw and processed data are available in the Gene Expression Omnibus (GEO) database [40] under the accession number GSE14920.

For real-time quantitative PCR (Q-PCR), total RNA samples (2 μg) were reverse-transcribed using SuperScript™ II reverse transcriptase (Invitrogen, Cergy Pontoise, France). Primers for SYBR Green assays are presented in Table 1. Amplifications were performed on an ABI Prism 7000 (Applied Biosystems, Courtaboeuf, France). Q-PCR data were normalized by TATA-box binding protein (TBP) mRNA levels and analyzed with DART-PCR [41].

2.5. Statistical analysis of transcriptomic data

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

2.6. DNA and shRNA constructs

The pCR3-mCAR construct expressing the murine CAR [43] and the pEGFP-CAR [44] plasmid expressing a fusion protein between the enhanced green fluorescent protein (EGFP) and the murine CAR were kindly provided by Dr. M. Negishi. The pLKO-SH1012, pLKO-SH767, pLKO-SH1134, pLKO-SH648 and pLKO-SH504 HIV-1-derived lentiviral constructs (Sigma, Lyon, France) were used to knockdown the expression of CAR. The pLVTHM construct [45] is a HIV1-derived lentiviral vector carrying the GFP gene under the

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	CATGAGTGTAAATCGTCTCA
Cyp3a11	NM_007818	TCACACACACAGTTGTAGGCAGAA	GTTTACGAGTCCCATATCGGTAGAG
Cyp4a14	NM_007822	TCAGTCTATTTCTGGTGCTGTTC	GAGCTCCTTGTCCTTCAGATGGT
Tbp	NM_013684	ACTTCGTGCAAGAAATGCTGAA	GCAGTTGTCCGTGGCTCTCT
Vnn1	NM_011704	ATGAGGTTTATGCCTTTGGAGC	CCACAGGTGCGTAAATTGGTAG
CYP2B6	NM_000767	GGCCATACGGGAGGCCCTTG	AGGGCCCCCTTGGATTTCCG
GAPDH	NM_002046	GGTCGGAGTCAACGGATTTGGTCG	CAAAGTTGTCATGGATGACC

control of the elongation factor 1-α promoter. The packaging system is composed of the psPAX2 and pMD2.G plasmids (http://tronolab.epfl.ch/). All constructs were verified by sequence analysis.

2.7. Cell culture and transfections

JWZ murine hepatic cells, also referred to as MuSH immortalized hepatocytes [46], were kindly provided by Dr. J.P. Gray. 293FT cells were purchased from Invitrogen (Cergy Pontoise, France). Both cell lines were cultured in DMEM supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and BFS (10%). The culture medium of JWZ cells was supplemented with dexamethasone (1 μM) and those of 293FT cells was supplemented with sodium pyruvate (1 mM) and non-essential amino acids (100 μM, Invitrogen, Cergy Pontoise, France). The pCR3-mCAR plasmid was transfected into the JWZ cells using lipofectamine 2000 according to the manufacturer's protocol. After 48 h, the transfected cells were submitted to geneticin selection for 10–15 days. Individual resistant clones were harvested, amplified and cryopreserved before being tested for CAR transcript expression using Q-PCR.

The pLVTHM and the pEGFP-CAR plasmids were transfected into the JWZ cells using lipofectamine 2000. After 48 h, the cells were transferred to LabTek chambered coverglasses (Nunc) for fluorescence analysis.

2.8. Fluorescence analysis

Cells adherent to the coverslips were fixed with 4% (w/v) paraformaldehyde (Sigma, Lyon, France) in PBS. Coverslips were then mounted onto slides using prolong gold antifade reagent (Invitrogen, Cergy Pontoise, France) and visualized under a ZEISS LSM 510 laser scanning confocal microscope.

2.9. Lentiviral shRNA vectors production and JWZ-CAR cell transduction

To produce the lentiviral particles, 293FT cells were cotransfected in suspension with the appropriate lentiviral shRNA or control vector and the packaging constructs using lipofectamine 2000. After 24 h the culture medium was replaced with fresh medium containing sodium butyrate (10 mM) known to improve vector titers. Viral supernatants were harvested 48 h after transfection, filtered (0.45 μm) and titrated on 293FT cells by serial dilutions and fluorescence-activated cell sorter (FACS) analysis for the ratio of GFP positive cells. The vectors harbouring the puromycin-resistance gene in place of the GFP gene were titrated according to Sigma recommendations. Viral titers of $\sim 10^5$ transforming units (TU)/ml were obtained. To isolate stable shRNA cell lines, JWZ-CAR (JWZ cells expressing active CAR) were infected with the viral vector stocks at a multiplicity of infection (MOI) of 1. Following puromycin selection (10–15 days) CAR and Cyp2b10 transcripts expression were measured by Q-PCR.

2.10. Primary culture of human hepatocytes

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

3. Results

3.1. Hepatic impact of DEHP: gene profiling in wild-type and PPARα-deficient mice

To evaluate the modulation by DEHP of hepatic NR pathways other than PPARα, we used a NR-dedicated cDNA macroarray (INR*Array* 01.4) to profile hepatic gene expression in wild-type and PPARα-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 eight 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 PPARα-deficient control mice. Interestingly, while DEHP (high dose) influenced 49 transcripts in wild-type mice, 16 modulations were recorded in PPARα-deficient livers, and the following four regulations occurred in both genotypes: acyl-Coenzyme A oxidase 1 (Acox1), aldehyde dehydrogenase 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 PPARα-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, PC1 captured a constitutive genotype effect and a PPARα-dependent effect of DEHP, both effects affecting similar genes. These genes are the longer vectors displaying a low angle with PC1 on the loading plot in Fig. 1B, such as Cyp4a10, Ech1, Scd1, Angptl4, Cyp4a14, Cdkn1, Acox, Peci, Apoa4 or Slco1a1. Most of these genes are well-known PPARα

Table 2
Liver gene expression modulations induced by DEHP treatment.

Gene description			Fold change		
Function and gene name	GenBankTM RefSeq	Symbol	PPARα$^{-/-}$ 200/Ctrl	Wild-type 200/Ctrl	Untreated PPARα$^{-/-}$/wt
Fatty acid metabolism					
Acetyl-Coenzyme A acyltransferase 1A	NM_130864	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-Coenyme 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		
Fatty acid transport					
ATP-binding cassette, subfamily 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	
Lipoprotein metabolism					
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
Fatty acid synthesis					
Elongation of very long chain fatty acids [FEN 1/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
Cholesterol synthesis					
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-methlylglutaryl-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	
Xenobiotic metabolism					
ATP-binding cassette, subfamily C (CFTR/MRP), member 2	NM_013806	Abcc2		1.4	
ATP-binding cassette, subfamily 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	1.6
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 transporter family), member 1	NH_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
Sulfotransferase 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		

Table 2 (*Continued*)

Gene description			Fold change		
Function and gene name	GenBankTM RefSeq	Symbol	PPARα$^{-/-}$ 200/Ctrl	Wild-type 200/Ctrl	Untreated PPARα$^{-/-}$/wt
Others					
Aminolevulinic acid synthase 1	NM_020559	Alas1	2.7	2.1	
Actin, beta, cytoplasmic	NM_007393	Actb		1.4	
Angiopoietin-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-Methylenetetrahydrofolate-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
Nuclear receptor					
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 INRA*rray* 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.

targets [49]. Remarkably, when considering PC2 alone, the impact of DEHP exposure (200 mg/(kg day)) was noticed for both genotypes (overall position of black circles and squares within the upper half of PC2). The loading plot (Fig. 1B) indicated that the following transcripts were co-ordinately increased by DEHP in both genotypes: Papss2, Cyp2b10, Gstt3, Abcc3, Nr1i3/CAR, Sult1d1, Cyp2c29 and Alas1. Strikingly, CAR itself and several downstream targets of CAR were found in this list. This raised the possibility that DEHP could recruit the CAR signalling pathway, *in vivo*, in the liver.

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 PPARα target genes which were expected to respond to the treatment. Both genes exhibited an altered constitutive expression in PPARα-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 PPARα-null mice (2.8-fold-increase) (Fig. 3A). For Cyp2c29, Cyp3a11 and Alas1, first, we noticed significantly lower constitutive expression levels in PPARα-null mice compared to wild-type mice (2.0-, 2.7- and 2.6-fold decrease, respectively) (Fig. 3B–D). Cyp2c29 transcript responded significantly to both doses of DEHP in wild-type mice and responded to the same extent to the higher dose of DEHP even in the PPARα-null mice (1.6-fold-changes in both genotypes) (Fig. 3B). Cyp3a11 displayed a modest, but significant, increase in wild-type mice only (×1.5, 200 mg/(kg day) DEHP) (Fig. 3C). Alas1 was up-regulated by DEHP, in both genotypes, in comparable proportions (Fig. 3D).

When we submitted the 88 genes in Table 2 to ingenuity pathways analysis (IPA), the CAR pathway was found to be significantly over-represented compared to the macroarray composition. Out of the 21 genes which undergo the influence of CAR, according to ingenuity knowledge base (Fig. 3E), 13 were spotted on our macroarray (genes in grey in Fig. 3E). 9 genes out of these 13 (asterisks in Fig. 3E), participated to PC2. This further supported the existence of a PPARα-independent response to DEHP.

Based on these initial results which strongly suggested the activation of the CAR signalling pathway by phthalates, we initiated a set of experiments to establish this proposal.

3.3. Establishment of hepatocyte cell lines expressing a functional CAR

As a prerequisite we had to develop a stable cell line expressing the functional CAR. An initial screening conducted on readily available hepatic cell lines, revealed that the JWZ (MuSH) [46] and the Hepa-I lines expressed standard levels of Retinoid X Receptor

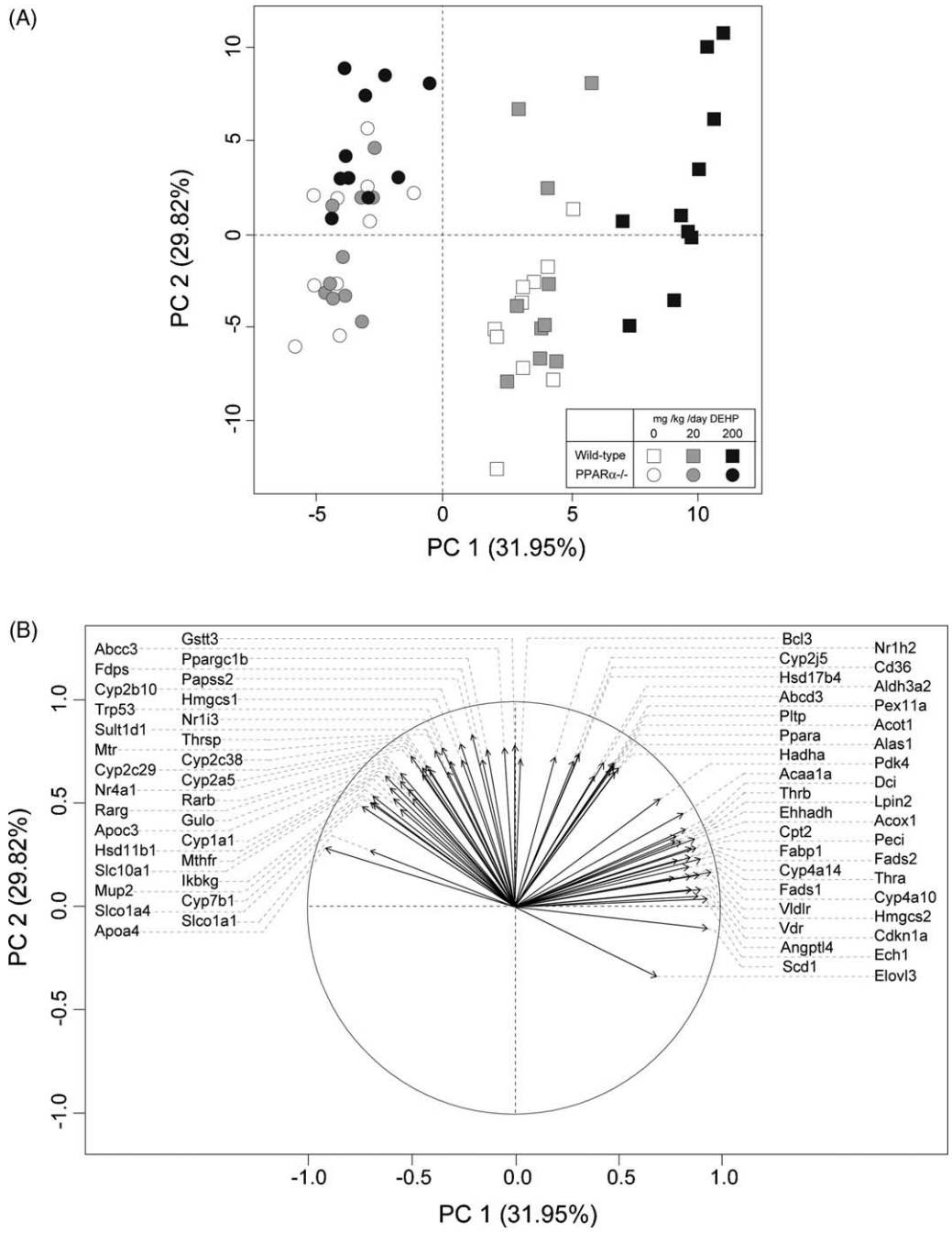

Fig. 1. Principal component analysis (PCA) of liver transcriptional signature. PCA was performed on a data set containing all 60 samples. 88 transcripts were initially analyzed for each sample. (A) Individual projection in PC1 and PC2 (first principal plan) is shown for liver of wild-type (square) and PPARα$^{-/-}$ 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).

(RXR), the heterodimeric partner of CAR, while CAR transcript was barely or not detectable (QPCR experiments, data not shown). Using the pCR3-mCAR expression vector, we established Hepa-I-CAR and JWZ-CAR cell lines which stably expressed the murine CAR nuclear receptor. Based on further functional test results (not shown), we selected the JWZ-CAR line for this study. In this line, the expression of the CAR transcript was 60-fold higher than in the parental JWZ cells (Fig. 4A). Consequently, this resulted in a 10-fold constitutive increase in Cyp2b10 mRNA expression, a known downstream target of CAR (Fig. 4A). This suggested a spontaneous, and at least partly, nuclear localization of the active CAR protein in our JWZ-CAR line; a pattern previously reported in HepG2 cells [44]. Indeed, we confirmed this assumption by expressing a CAR–green fluorescent protein (GFP) fusion protein. The transfection of native JWZ cells with a plasmid expressing GFP alone (pLVTHM) led to a conventional cytoplasmic localization of the fluorescence (Fig. 4B, upper lane pictures), while the same experiment with the EGFP-CAR fusion protein revealed a fluorescence which was restricted to the nucleus (Fig. 4B, lower lane pictures). Thus, in our JWZ-CAR model, CAR is localized in the nucleus and its constitutive activity increases the expression of its prototypic target gene Cyp2b10.

For further functional investigations, we developed a panel of JWZ-CAR sublines stably expressing shRNAs targeting CAR. Out

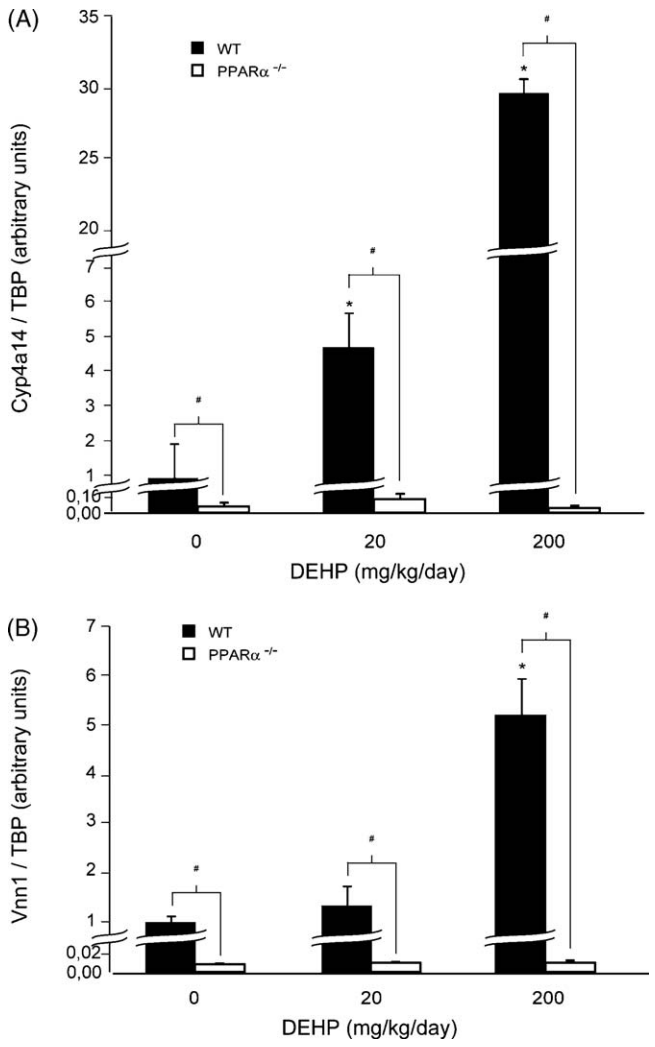

Fig. 2. Effect of DEHP administration on hepatic mRNA levels of PPARα dependent genes. Relative expressions of Cyp4a14 (A) and Vanin1 (B) mRNA were determined using Q-PCR normalized to the expression of TBP. Values shown are the mean ± S.E.M. ($n = 10$ for each group). Significant differences were analyzed 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 "#". $P < 0.05$.

of the five shRNA-carrying lentiviral vectors tested, two constructs displayed a convincing efficacy to lower CAR expression. We measured a reduction of 63% and 69% of CAR mRNA level with pLKO-SH1012 and pLKO-SH767, respectively (Fig. 4C). This translated into the parallel reduction of the constitutive level of the Cyp2b10 mRNA by 69% and 70% (Fig. 4C).

3.4. DEHP regulates gene expression through the CAR signalling pathway

Firstly, we evaluated the response of the CAR pathway to an agonist (TCPOBOP [52]) or an inverse agonist (androstenol [53]) in the JWZ-CAR recombinant cells. TCPOBOP doubled the level of Cyp2b10. Androstenol, which alone reduced Cyp2b10 constitutive expression, abolished Cyp2b10 induction by TCPOBOP (Fig. 5A, columns 1–4).

The *in vivo* effect of DEHP on Cyp2b10 hepatic transcript was reproduced (Fig. 5A) in the recombinant JWZ-CAR cell line. Indeed, the incubation of the cells with 100 μM of DEHP resulted in a 2-fold increase of Cyp2b10 mRNA, equivalent to that observed with TCPOBOP (Fig. 5A, column 5). Similarly, androstenol abolished the induction of Cyp2b10 by DEHP, thus supporting the involvement of CAR in this regulation (Fig. 5A, column 6). In our JWZ-CAR cell line MEHP (100 μM), the main DEHP metabolite, was unable to increase Cyp2b10 mRNA (Fig. 5B).

Finally, using the SH1012 and SH767 cell lines expressing CAR-targeting shRNAs, we showed that a ~60% reduction in CAR expression led to a loss of the ability to modulate Cyp2b10 mRNA level by TCPOBOP, androstenol, or DEHP (Fig. 5C). Thus, this established that, like TCPOBOP, DEHP regulates the prototypic Cyp2b10 target by activating CAR.

3.5. DEHP regulates CYP2B6 expression in human primary hepatocytes

To evaluate whether the activation of CAR by DEHP could be extrapolated to humans, we tested the effects of DEHP on CYP2B6 expression in two independent cultures of human primary hepatocytes. Consistently, DEHP dose-dependently increased the expression of CYP2B6 at 50 and 100 μM of DEHP in both primary hepatocyte cultures (Fig. 6).

4. Discussion

Human exposure to phthalates inevitably occurs due to their use in plastics and consumer products [2]. In the adult, the main concerns associated to phthalate exposure are linked to hepato-carcinogenesis [10], endocrine [9,25] and metabolic disruption [27]. Despite many studies, the mechanisms involved in these effects remain poorly understood.

In this study, we confirm the responsiveness of PPARα pathway to DEHP (activation even at the 20 mg/(kg day) low dose, Fig. 2A). And when abrogating the dominant signal elicited by PPARα using PPARα-null mice, we revealed the underlying activation of the hepatic CAR pathway (Table 2 and Figs. 1 and 3). The activation of CAR by DEHP was then demonstrated using a CAR-expressing cell line which was exposed either to a CAR inverse agonist (androstenol, Fig. 5A) or to shRNAs targeting CAR (Fig. 5C).

Consistent with our finding, it was recently reported that DEHP may activate Cyp2b10 promoter, but this was done only with a gene reporter system [54]. Our study is the first report on DEHP activation of Cyp2b10 gene both *in vitro* and *in vivo*. It also provides the first *in vitro* demonstration that CAR is involved in the regulation of Cyp2b10 by DEHP, which could otherwise be attributed to PXR. CAR activation by phthalates may not be restricted to mice species and to DEHP since an other phthalate, the Di-n-butyl phthalate (500 mg/(kg day)) was shown to increase the CAR-target Cyp2b1 transcript in late rat embryos (E:19) [55]. Interestingly, another group has very recently reported that the CAR2 splice variant of human CAR is activated by DEHP [56]. Similar to their observations using *in vitro* reporter assays [56], we show here that MEHP, the main DEHP metabolite does not induce Cyp2b10 in our JWZ-CAR cell line. It remains to be established whether DEHP or one if its metabolite other than MEHP directly binds to mouse and/or human CAR.

CAR (Nr1i3) was first described as a xenobiotic-sensing nuclear receptor that regulates the expression of hepatic enzymes and transporters involved in xenobiotic metabolism [57]. It is closely related to PXR (Nr1i2) and these receptors share both ligands [58] and target genes [59]. Indeed, DEHP and MEHP also activate mouse and human PXR [34,60] which result in CYP3A induction. *In vivo*, we observed a higher induction by DEHP of Cyp2b10 expression (Fig. 3A) compared to Cyp3a11 (Fig. 3C), suggesting that the CAR pathway is at least as responsive as the PXR pathway to DEHP.

Importantly, the xenobiotic metabolizing systems induced by CAR and PXR are also involved in the metabolism of endogenous molecules including steroid and thyroid hormones. Both

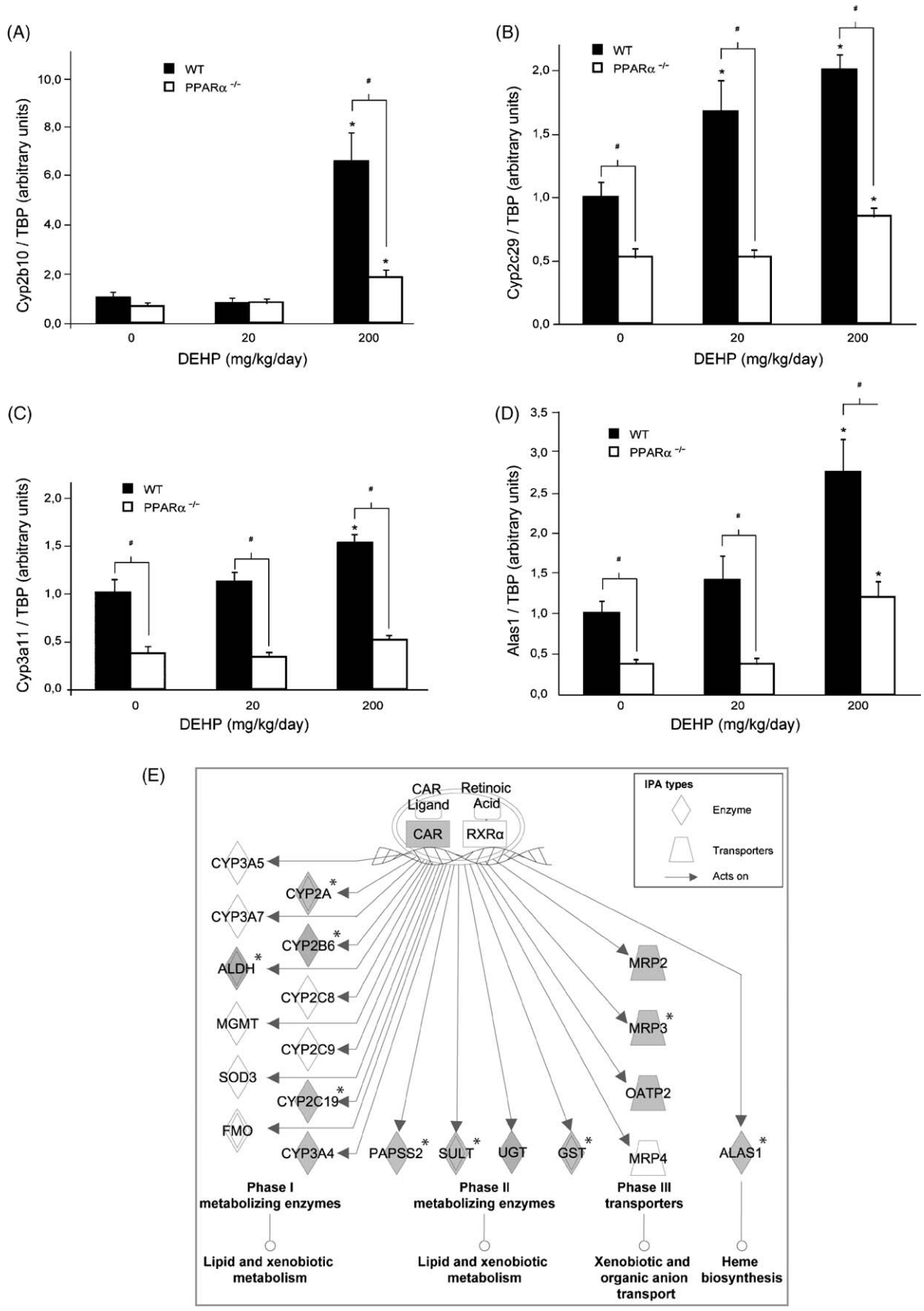

Fig. 3. Effect of DEHP administration on hepatic mRNA levels of CAR target genes. Relative expressions of Cyp2b10 (A), Cyp2c29 (B), Cyp3a11 (C) and Alas1 (D). mRNA were determined using Q-PCR normalized to the expression of TBP. Values shown are the mean ± S.E.M. (n = 10 for each group). Significant 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 (*).

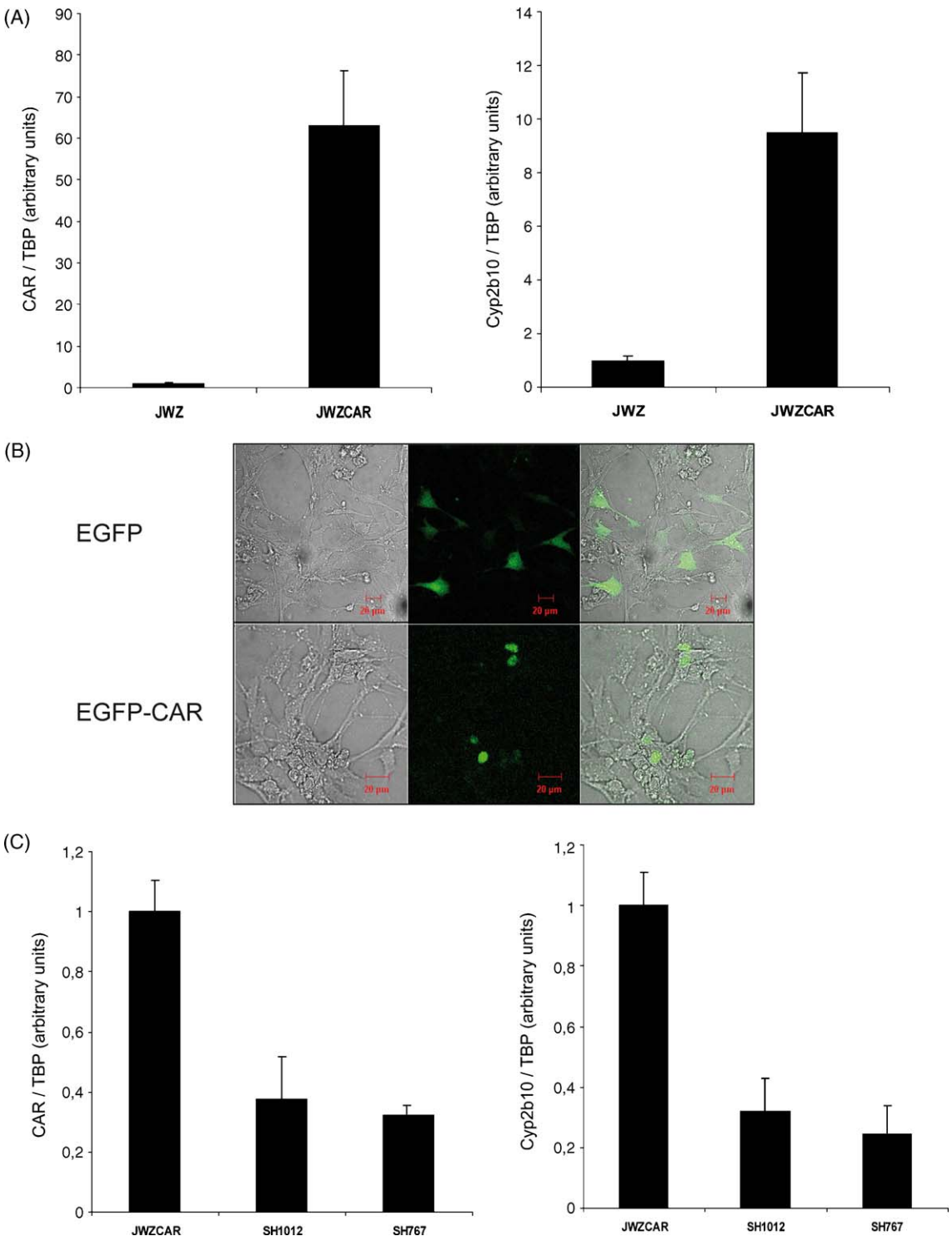

Fig. 4. New murine hepatocyte cell line expressing the CAR nuclear receptor and CAR directed shRNA. (A) Q-PCR results of relative expressions of CAR and Cyp2b10 in the parental JWZ cell line and the newly established JWZCAR cell line. Values shown are the mean ± S.E.M. (n = 3 for each cell line). (B) 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 an 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.

receptors protect the body from the deleterious accumulation of bilirubin [61,62] and bile acids [63] through hepatic induction of several enzyme genes. Additionally, many CAR and PXR target genes, including CYP3A and CYP2B, are involved in steroid hormone metabolism and their induction could contribute to the endocrine disruption activities of drugs and contaminants [64,65]. CYP2B6 induction following DEHP exposure of human hepatocytes confirmed a possible extrapolation to humans of this DEHP-mediated CAR activation observed in mice (Fig. 6 [56]). This induction could contribute to increased hepatic testosterone metabolism, leading to a reduction of circulating testosterone levels as observed in workers exposed to high levels of phthalates [9].

Similarly, the CAR-dependent induction of genes involved in hepatic phase II xenobiotic metabolism is responsible for increased thyroid hormone metabolism [66]. Consistently, CAR-deficient

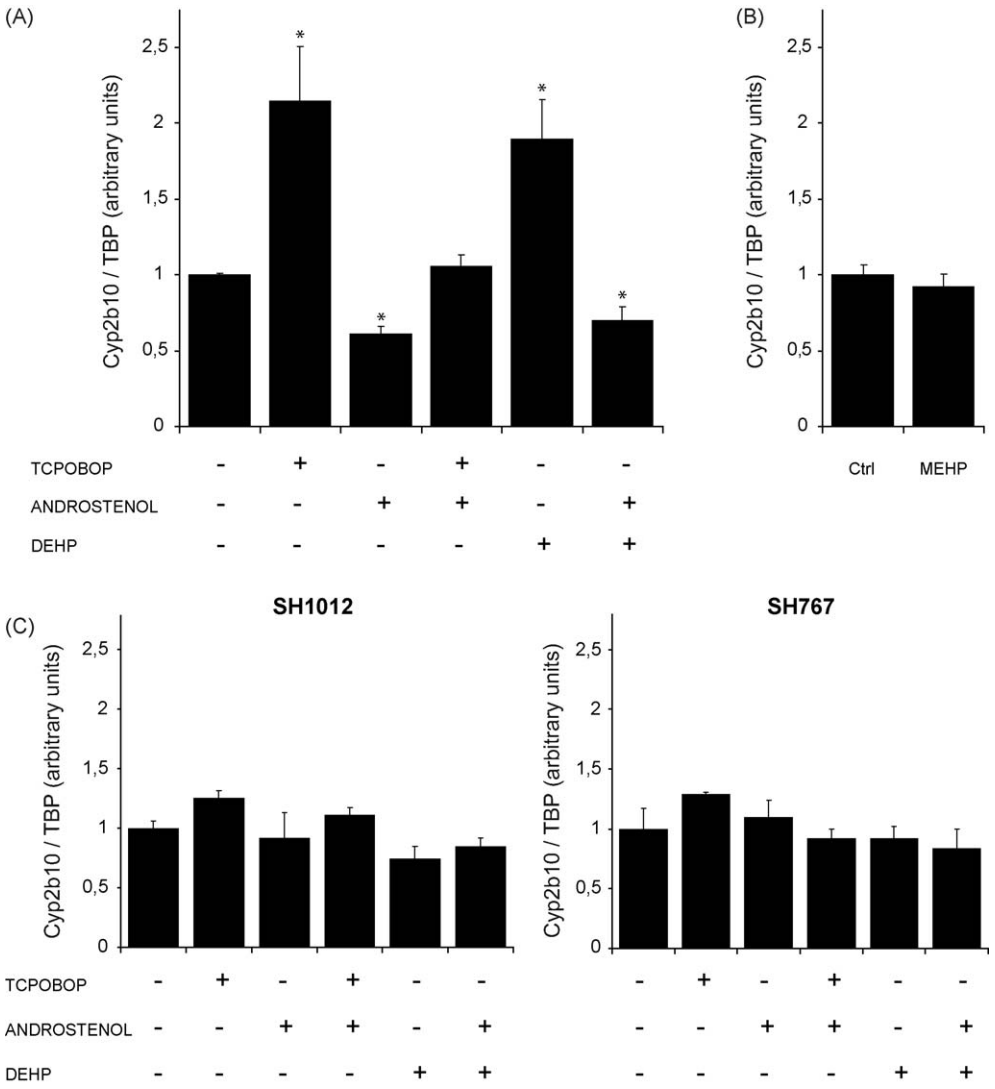

Fig. 5. CAR dependent effect of DEHP and MEHP on Cyp2b10 mRNA levels. Cells were incubated for 48 h in the presence of DMSO, TCPOBOP (200 nM), androstenol (5 μM), DEHP (100 μM), MEHP (100 μM) or a mix of TCPOBOP and androstenol or DEHP and androstenol. Cyp2b10 mRNA were then quantified by QPCR. Values shown are the mean ± S.E.M. ($n = 3$ for each cell treatment). (A) Cyp2b10 mRNA modulation after TCPOBOP, androstenol 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 μM) does not induce Cyp2b10 expression in the JWZCAR cell line. (C) No more significant Cyp2b10 mRNA modulation after treatment with TCPOBOP, androstenol or DEHP in the CAR-knockdown cell lines (SH1012 and SH767).

mice loose more weight than wild-type controls during caloric restriction, likely due to a higher metabolic rate driven by higher thyroid hormone levels [67]. Interestingly, recent studies have suggested an inverse association between phthalate exposure and thyroid hormone levels in men [68] and pregnant women [69]. Given our observations, it is conceivable that CAR activation by DEHP contributes to a reduction in thyroid hormones which could favour a positive energy balance and weight gain in a context of high caloric intake. Accordingly, we have observed that high DEHP exposure (1100 mg/(kg day) for 2 weeks) led to a significant reduction in plasma total T4 (data not shown). CAR and PXR are also increasingly recognized as important regulators of genes involved in lipid and glucose homeostasis (for review: [70,71]). In particular, CAR activation reduces the expression of critical genes involved in fatty acid oxidation [72], bile acid synthesis and gluconeogenesis [73]. Further studies should evaluate the potential role of these regulations in mediating metabolic disruption by phthalates in light of their potential to activate both PXR and CAR.

Finally, CAR also mediates the hepatocarcinogenic effects of its xenobiotic ligands [74], suggesting that it may contribute to

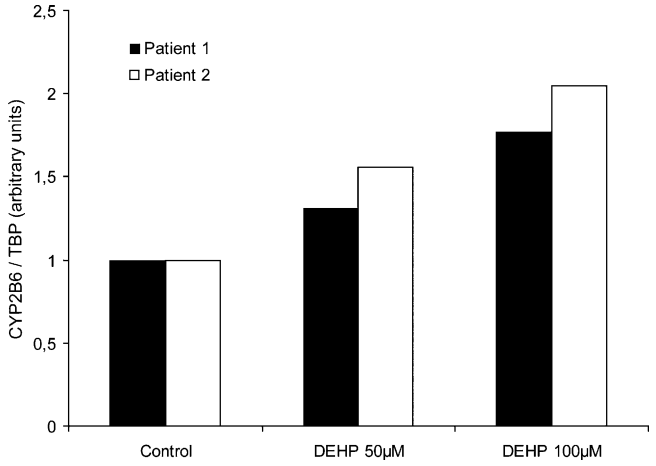

Fig. 6. Effect of DEHP on CYP2B6 mRNA level in human primary hepatocytes. Q-PCR results of relative expressions of CYP2B6 in primary hepatocytes derived from 2 patients (patient1 and patient 2) treated with 2 concentrations of DEHP (50 and 100 μM).

PPARα-independent hepatocarcinogenesis [13] following high-dose DEHP exposure. Indeed, we have observed that high DEHP exposure (1100 mg/(kg day), 14 days) in mice induces the hepatic expression of Mdm2 (2.1-fold, data not shown), a direct CAR target gene that contributes to hepatocyte proliferation [74].

Overall, our results demonstrate that DEHP activates the CAR pathway which results in the induction of several hepatic target genes *in vivo*. This adds DEHP to the growing list of environmental contaminants activating CAR [54,75–77], further stressing the need to evaluate the specific role of this NR in mediating the array of deleterious carcinogenic, endocrine and metabolic effects associated with exposure to these compounds and their mixtures.

Disclosure statement

The authors have nothing to disclose.

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