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Transcriptional Activation of PPARα by Phenobarbital in the Absence of CAR and PXR

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Abstract: The nuclear receptors CAR (constitutive androstane receptor) and PXR (pregnane X receptor) mediate the effects of phenobarbital on gene transcription. To investigate the relative contribution of these nuclear receptors to the expression of specific genes we studied the effect of phenobarbital in livers of wild type, CAR$^{-/-}$, PXR$^{-/-}$ and CAR/PXR$^{-/-}$ knockout mice. Spotted Steroltalk v1 cDNA arrays were applied containing probes for genes involved in drug metabolism, sterol biosynthesis, steroid synthesis/transport and heme synthesis. In the absence of CAR and PXR, phenobarbital unexpectedly induced mRNAs of several nuclear receptors, including PPARα and its target genes Cyp4a10 and Cyp4a14. Interestingly, in primary cultures of hepatocytes isolated from CAR/PXR$^{-/-}$ knockout mice, phenobarbital increased HNF-4α levels. In further experiments in these hepatocyte cultures we provide evidence that phenobarbital directly induces transcription of the PPARα gene via its HNF-4α response element, and indirectly by lack of inhibitory crosstalk of AMPK, CAR and PXR with HNF-4α. Our results provide further insight into CAR and PXR-independent effects of phenobarbital and the crosstalk between different nuclear receptor signaling pathways.

Keywords: Phenobarbital; microarrays; CAR$^{-/-}$; PXR$^{-/-}$; CAR/PXR$^{-/-}$; PPARα; HNF-4α; AMPK

Introduction

Phenobarbital (PB) is a hypnotic and antiepileptic drug that causes numerous effects in the liver including hypertrophy, proliferation of the smooth endoplasmatic reticulum and tumor promotion.[1,2] Within this pleiotropic response phenobarbital induces or represses genes involved in drug metabolism and transport, sterol, bile acid, lipid and energy metabolism.[3−5] The transcriptional activation of many of these genes by phenobarbital is mediated by CAR (constitutive androstane receptor, NR1I3) and by PXR (pregnane X receptor, NR1I2). Whereas phenobarbital probably directly binds to and activates PXR, it does not bind to CAR but triggers CAR translocation to the nucleus from a cytosolic multiprotein complex.[6,7] Recent studies in our laboratory have revealed that the induction of genes by phenobarbital in mouse and human liver requires the activation of AMP-

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activated protein kinase (AMPK) by a process involving activation of its upstream kinase LKB1.[8−10]

To investigate the relative role of CAR and PXR in the induction response, microarrays were generated containing genes which are known to be regulated with these or related nuclear receptors (genes involved in drug metabolism, cholesterol biosynthesis, sterol synthesis/transport, heme synthesis).[11] Samples from livers of wild type and CAR$^{-/-}$, PXR$^{-/-}$ or CAR/PXR$^{-/-}$ knockout mice were tested after treatment with phenobarbital and other inducers for gene expression within the European Framework 6 project Steroltalk (http://www.steroltalk.net). Results from these experiments unexpectedly revealed that if CAR and PXR are deleted, phenobarbital increases the expression of several other nuclear receptors, for example PPARα.

In the present study we explore the mechanism by which phenobarbital leads to induction of PPARα and its target genes in cultures of hepatocytes from mice deficient in PXR and CAR. Our experiments suggest a major role of HNF-4α in the transcriptional regulation of PPARα under these conditions.

Methods

Chemicals. Phenobarbital was purchased from Merck (Dietikon, Switzerland) and compound C (Comp C) from Calbiochem (Laufelfingen, Switzerland). AICAR was from Toronto Research (North York, ON, Canada). All other chemicals were obtained from Sigma (Buchs, Switzerland) or Invitrogene (Carlsbad, CA).

Animals. The first breeding pair of CAR knockout mice (C57BL/6J background) was a generous gift of Dr. D. D. Moore from the Department of Molecular and Cellular Biology, Baylor College of Medicine (Houston, TX).[12] The PXR knockout mice (C57BL/6J background) were obtained from a colony maintained at the Biozentrum, which was started with animals kindly provided by Dr. S. A. Kliewer and GlaxoSmithKline, Research Triangle Park, NC.[13] CAR/PXR$^{-/-}$ mice were generated by crossbreeding of CAR and PXR knockout animals.

RNA Extraction and Quantitative RT-PCR. Total RNA was isolated from mouse liver and hepatocytes using TRIzol reagent (Invitrogen, Carlsbad, CA). Liver cells were homogenized in 1 mL of TRIzol reagent, and total RNA was extracted according to the manufacturer's instructions. RNA was purified using phase Lock Gel Heavy tubes (Eppendorf, Westburry, NY). The quality of RNA was determinated with an Agilent 2100 Bioanalyzer (Santa Clara, CA). Reverse transcription (RT) reactions were carried out in a final volume of 20 μL with 2 μg of mouse total RNA, 0.45 μg of random hexamer, 20 U of RNasin, 0.5 μM dNTP mix, and 200 U of SuperScript II Reverse Transcriptase at 42 °C for 1 h (Invitrogen, Carlsbad, CA). The cDNA product was treated to remove RNA and the oligo DNA mixture with QIAquick PCR purification kit (Promega, Madison, WI). The Taqman reaction was carried out with a model ABI7000 DNA sequence detection analyzer in a total volume of 25 μL using reverse and forward primers designed by Operon (Cologne, Germany). Mouse cDNA was analyzed with FAM labeled Taq probes (Microsynth GmbH, Balgach, Switzerland) using AmpliTaq DNA Polymerase (TaqMan universal PCR Master Mix, No AmperaseUNG, Applied Biosystems, Branchburg, NJ). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and cyclophilin (CYCLO) were used as housekeeping genes.

Microarray Hybridization. Sterolgene v1 cDNA microarrays were prepared as described by Rezen et al..[11] Six mice per treatment group were compared to pooled controls. 10 μg of RNA was reverse transcribed along with Amersham Lucidea Universal Scorecard (GE Healthcare UK, Buckinghamshire, U.K.) to cDNA using aminoallyl labeling. The aminoallyl labeled cDNA was conjugated to Cy3 (control) or Cy5 (treated) dyes. Efficiency of the cDNA labeling (base to dye ratio) was measured with a Nanodrop ND-1000 instrument (Wilmington, DE). Hybridization was carried out overnight and after washing arrays were scanned using the Affimetrix 428 Array Scanner and Jaguar 2.0 software (Affymetrix, Santa Clara, CA). Images were analyzed using GenePix Pro 6.0 software (Molecular Devices Corporation, Sunnyvale, CA).

Preparation and Culture of Primary Mouse Hepatocytes. Primary hepatocytes of CAR/PXR$^{-/-}$ knockout mice were prepared by a two step collagenase method.[14] Liver cells were plated at a density of 3×10^5 cells/well on 12-well plates coated with collagen and maintained in

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medium described by Ferrini and co-workers.[15] Hepatocytes were exposed to PB or AICAR and cells were collected in TRIzol reagent for expression analysis.

Transient Transfection of Primary Hepatocytes. Cells were plated in 12-well plates at a cell density of 1.5×10^5 per well. After 4 h cells were transiently transfected in Optimem I Reduced Serum Medium (Invitrogen, Carlsbad, CA) with various plasmids (pSG5-empty, pSG5-mCAR, pSG5-mPXR, pEF6-empty, pEF6-HNF-4α) using Lipofectamine (Invitrogen, Carlsbad, CA) or using viral transfection (ad-B-Gal, ad-CA-AMPKα1). After 12 h cells were treated for 48 h with inducers. Total RNA was isolated for further measurement.

Data Analysis. Data from microarray experiments were normalized using LOWESS fit[16] to firefly luciferase (Promega, Madison, WI) spike in controls and housekeeping genes according to their average intensity using Orange[17] widget for normalization of focused microarrays. Data have been submitted to GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo) under accession code GSE12489. Statistical analysis was performed in R using LIMMA package[18] by controlling type I error at $\alpha = 0.05$.

All other experiments were conducted in triplicate or quadruplicate, and means ± standard deviations were determined. Statistical comparisons among treatment groups were determined using a Student's two-tailed *t* test by controlling type I error at $\alpha = 0.05$.

Results

Transcriptome Analysis in Phenobarbital Treated Mice. Treating mice lacking the typical phenobarbital activated nuclear receptors, such as CAR and PXR with phenobarbital, causes increased expression of FXR, PGC-1α, PPARα or decreased expression of LXR*β* and other nuclear receptors (Table 1A,B). Experiments with more selective CAR (TCPOBOP) and PXR inducers (PCN) were also performed, and all three inducers were tested on wild type, $CAR^{-/-}$, $PXR^{-/-}$ and $CAR/PXR^{-/-}$ animals. Results from other treatments and other transgenic mice are accessible in GEO under accession codes GSE12509 (TCPOBOP) and GSE12537 (PCN).

Induction of Nuclear Receptors and PPARα Receptor Target Genes in Vivo. To investigate the effect of a single ip injection of phenobarbital on PPARα in different transgenic mice, the mRNA levels of PPARα and PPARα target genes were measured, such as Cyp4a10 and Cyp4a14. Classical phenobarbital target genes, such as Cyp2b10, were also monitored. PPARα mRNA did not change after phenobarbital treatment in wild type mice, but its level was significantly increased in the single knockout and $CAR/PXR^{-/-}$ mice (Table 2). CYP4a10 mRNA was also increased by 5-fold in mice lacking the PXR and CAR receptors, and almost 3-fold in $CAR^{-/-}$ mice, but not in wild type mice. Cyp4a14 mRNA expression was upregulated by 7-fold in $CAR/PXR^{-/-}$ mice after phenobarbital treatment, and Cyp2b10 mRNA was as expected, largely increased in wild type and in $PXR^{-/-}$ animals.

Induction of PPARα and PPARα Target Genes in Vitro. To investigate if the observed changes in PPARα also occur in vitro, primary $CAR/PXR^{-/-}$ hepatocytes were treated with 1 mM phenobarbital for 24 and 48 h and after that mRNA levels of PPARα and its target genes were measured (Figure 1). After 48 h induction time, higher induction was found, therefore this time point was used in our further studies. In these experiments, PB elevates PPARα mRNA levels by 3-fold and elevates mRNA levels of Cyp4a10 (2-fold) and Cyp4a14 (5-fold).

Induction of PPARα Is CAR and PXR Dependent. Since PPARα and its target genes are not induced by PB in wild type animals, we wished to test if this induction is directly related to the absence of these nuclear receptors. $CAR/PXR^{-/-}$ cells were transfected with expression constructs of mCAR and/or mPXR and treated with 1 mM PB for 48 h. Total RNA was isolated from these cells and analyzed with RT-PCR. Levels of PPARα (Figure 2A) are induced (4-fold) with phenobarbital in mock transfected PXR/CAR deficient cells, but there is no significant elevation after phenobarbital treatment, if mCAR or mPXR is retransfected. CYP4a10 is induced highly after phenobarbital in the mock transfected (5-fold) and in the mPXR transfected (15-fold) cells (Figure 2B). The induction is reversed by mCAR or simultaneous mCAR/mPXR transfection. These observations suggest a direct inhibitory role of CAR in suppressing PPARα induction by PB.

AMPK Activation Inhibits PPARα Induction by Phenobarbital. PB induction of PPARα was impaired by 0.2 mM AICAR (Figure 3A), which suggests that activation of AMPK suppresses the observed action of PB. Compound C (Comp C), a specific inhibitor of AMPK activity, had no significant effect on PB induction in $CAR/PXR^{-/-}$ hepatocytes (Figure 3B). Morever, when cells were transfected with the constitutively active form of AMPK (ad-CA-AMPKα1) and treated with PB, there was no PPARα induction (Figure 3C). Thus, activation of AMPK with AICAR or with overexpression of active AMPK abolished the effect of phenobarbital on PPARα.

HNF-4α Regulates PPARα Receptor Expression in $CAR/PXR^{-/-}$ Cells. Pineda Torra and co-workers reported a functional HNF-4α binding site of the 5′ flanking region

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Table 1. Selection of Differentially Expressed Genes in Wild Type and CAR/PXR$^{-/-}$ Mouse Livers after Phenobarbital Treatment as Detected by the Sterolgene v1 cDNA Microarray[a]

log 2 ratio	GenBank acc no.	gene symbol	gene name
		(A) Wild Type, Phenobarbital Treatment	
		Nuclear Receptors Superfamily	
−0.67	**BC028890**	**Cebpa**	**CCAAT/enhancer binding protein (C/EBP), alpha**
−0.49	**NM_008904**	**Ppargc1a**	**peroxisome proliferative activated receptor, gamma, coactivator 1 alpha**
		Cytochrome P450 Superfamily: Xenobiotic Metabolism	
1.89	AK028103	Cyp2b10	cytochrome P450, family 2, subfamily b, polypeptide 10
1.64	**NM_007813**	**Cyp2b13**	**cytochrome P450, family 2, subfamily b, polypeptide 13**
0.73	**NM_007819**	**Cyp3a13**	**cytochrome P450, family 3, subfamily a, polypeptide 13**
0.91	NM_017396	Cyp3a41	cytochrome P450, family 3, subfamily a, polypeptide 41
		Cytochrome P450 Superfamily: Metabolism of Endogenous Compounds	
−0.52	NM_010007	Cyp2j5	cytochrome P450, family 2, subfamily j, polypeptide 5
−0.52	U62295	Cyp2j6	cytochrome P450, family 2, subfamily j, polypeptide 6
−0.66	BC038810	Cyp7b1	cytochrome P450, family 7, subfamily b, polypeptide 1
−0.84	NM_009995	Cyp21a1	cytochrome P450, family 21, subfamily a, polypeptide 1
		Transporters	
−0.58	AY195868	Slco1a1	solute carrier organic anion transporter family, member 1a1
		Cholesterol Biosynthesis	
−0.59	BC031813	Cyp51	cytochrome P450, family 51
−0.62	BC019797	Dhcr24	24-dehydrocholesterol reductase
−0.76	NM_134469	Fdps	farnesyl diphosphate synthase 1
−0.90	NM_145942	Hmgcs1	3-hydroxy-3-methylglutaryl-coenzyme A synthase 1
−0.62	NM_010191	Fdft1	farnesyl diphosphate farnesyl transferase 1, squalene synthase
−0.62	BC024132	Sc5d	sterol-C5-desaturase homologue
		Heme Metabolism	
0.78	**NM_020559**	**Alas1**	**aminolevulinic acid synthase 1**
		(B) CAR/PXR$^{-/-}$, Phenobarbital Treatment	
		Nuclear Receptors Superfamily	
−0.39	NM_009380	Thrb	thyroid hormone receptor beta
0.48	**NM_011144**	**Ppara**	**peroxisome proliferator activated receptor alpha**
−0.46	NM_011146	Pparg	peroxisome proliferator activated receptor gamma
0.66	NM_011281	Rorc	RAR-related orphan receptor gamma
−0.41	**NM_009473**	**Nr1h2**	**nuclear receptor subfamily 1, group H, member 2**
0.54	**NM_009108**	**Nr1h4**	**nuclear receptor subfamily 1, group H, member 4**
−0.40	NM_011306	Rxrb	retinoid X receptor beta
−0.44	NM_009697	Nr2f2	nuclear receptor subfamily 2, group F, member 2
−0.37	NM_139051	Nr5a1	nuclear receptor subfamily 5, group A, member 1
0.41	**NM_008904**	**Ppargc1a**	**peroxisome proliferative activated receptor, gamma, coactivator 1 alpha**
		Cytochrome P450 Superfamily: Xenobiotic Metabolism	
−0.75	NM_009992	Cyp1a1	cytochrome P450, family 1, subfamily a, polypeptide 1
−0.59	NM_009993	Cyp1a2	cytochrome P450, family 1, subfamily a, polypeptide 2
0.43	BC011233	Cyp2a4	cytochrome P450, family 2, subfamily a, polypeptide 5
0.43	**NM_007819**	**Cyp3a13**	**cytochrome P450, family 3, subfamily a, polypeptide 13**
		Cytochrome P450 Superfamily: Metabolism of Endogenous Compounds	
−0.47	NM_010007	Cyp2j5	cytochrome P450, family 2, subfamily j, polypeptide 5
1.11	NM_007823	Cyp4b1	cytochrome P450, family 4, subfamily b, polypeptide 1
−0.31	NM_022434	Cyp4f14	cytochrome P450, family 4, subfamily f, polypeptide 14
−0.66	BC038810	Cyp7b1	cytochrome P450, family 7, subfamily b, polypeptide 1
1.57	NM_010012	Cyp8b1	cytochrome P450, family 8, subfamily b, polypeptide 1
0.65	NM_007809	Cyp17a1	cytochrome P450, family 17, subfamily a, polypeptide 1
−0.42	BC049147	Cyp20a1	cytochrome P450, family 20, subfamily A, polypeptide 1
−0.81	NM_007811	Cyp26a1	cytochrome P450, family 26, subfamily a, polypeptide 1

Table 1. Continued

log 2 ratio	GenBank acc no.	gene symbol	gene name
0.34	NM_024264	Cyp27a1	cytochrome P450, family 27, subfamily a, polypeptide 1
−1.21	NM_010010	Cyp46a1	cytochrome P450, family 46, subfamily a, polypeptide 1
−0.73	BC031813	Cyp51	cytochrome P450, family 51
		Transporters	
−0.58	AF323659	Abcg1	ATP-binding cassette, subfamily G (WHITE), member 1
−0.54	**NM_031884**	**Abcg5**	**ATP-binding cassette, subfamily G (WHITE), member 5**
−0.68	**NM_026180**	**Abcg8**	**ATP-binding cassette, subfamily G (WHITE), member 8**
−1.05	NM_011076	Abcb1a	ATP-binding cassette, subfamily B (MDR/TAP), member 1A
0.48	NM_008830	Abcb4	ATP-binding cassette, subfamily B (MDR/TAP), member 4
−0.48	**NM_021022**	**Abcb11**	**ATP-binding cassette, subfamily B (MDR/TAP), member 11**
0.33	**BC021154**	**Slc10a1**	**solute carrier family 10, member 1**
		Cholesterol Biosynthesis	
−1.06	NM_019811	Acss2	acyl-CoA synthetase short-chain family member 2
−0.36	NM_009338	Acat2	acetyl-coenzyme A acetyltransferase 2
0.49	NM_009692	Apoa1	apolipoprotein A-I
−0.76	NM_009890	Ch25h	cholesterol 25-hydroxylase
−0.43	BC006854	Dhcr7	7-dehydrocholesterol reductase
0.52	BC019797	Dhcr24	24-dehydrocholesterol reductase
−0.62	NM_007898	Ebp	emopamil binding protein (sterol C7,8-isomerase)
0.59	NM_008255	Hmgcr	3-hydroxy-3-methylglutaryl-coenzyme A reductase
0.32	NM_177960	Idi1	isopentenyl-diphosphate delta isomerase
0.38	NM_021460	Lipa	lysosomal acid lipase A
−0.48	NM_145554	Ldlrap1	low density lipoprotein receptor adaptor protein 1
−0.67	BC019945	Nsdhl	NAD(P) dependent steroid dehydrogenase-like
−0.57	AK054470	Star	steroidogenic acute regulatory protein
−0.66	BC024132	Sc5d	sterol-C5-desaturase homologue
		Heme Metabolism	
0.90	**NM_020559**	**Alas1**	**aminolevulinic acid synthase 1**

[a] Groups of 6 mice were treated for 12 h with 100 mg/kg phenobarbital and array hybridizations based on liver RNAs were performed as described in experimental procedures. Log ratios represent gene expression changes of the treated animals compared to untreated controls. Genes and expression changes marked with bold are confirmed with RT-PCR (relative to GPDH, $p < 0.05$).

Table 2. RT-PCR Measurements of Selected Genes in PB-Treated Wild Type and Transgenic Mice Compared to Untreated Animals[a]

gene symbol	wild type	CAR$^{-/-}$	PXR$^{-/-}$	CAR/PXR$^{-/-}$
PPARa	1.0 ± 0.2	1.6 ± 0.5*	1.8 ± 0.6*	2.2 ± 0.7*
CYP4a10[a]	1.3 ± 0.6	2.7 ± 0.9*	1.2 ± 0.9	5.2 ± 3.4*
CYP4a14[a]	1.1 ± 0.5	1.2 ± 1.6	2.1 ± 3.4	7.4 ± 3.4*
CYP2b10	2566.7 ± 548.5*	0.2 ± 0.1*	312.0 ± 56.0*	2.7 ± 1.9*

[a] These genes are not present on the SteroIgene v1 microarray. Average fold changes ± SD of mRNA expression are represented (relative to cyclophilin, *$p < 0.05$). The same samples were used as for microarray experiments shown in Table *1*.

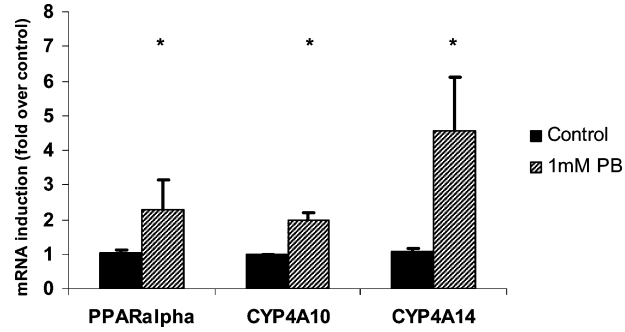

Figure 1. In vitro induction of PPARα and PPARα target genes by phenobarbital. Primary hepatocytes from CAR/PXR$^{-/-}$ animals were treated with 1 mM phenobarbital, and after 48 h, total RNA was isolated as described. RT-PCR Taqman reactions were carried out with specific primers for PPARα, Cyp4a10 and Cyp4a14. RT-PCR data are calculated relative to cyclophilin and are represented as fold over control. *$p < 0.05$.

in human PPARα.[19] Comparative sequence analysis with ENSEMBL revealed the corresponding, almost identical DR1 responsive element for HNF-4α in the mouse gene. In other species like *Rattus norvegicus* or *Macacus Malutta* this sequence was not detected (Figure 4A).

In primary hepatocytes from CAR/PXR$^{-/-}$ mice, HNF-4α mRNA increased 4-fold within 3 h of treatment with phenobarbital, but the elevation did not persist after 48 h

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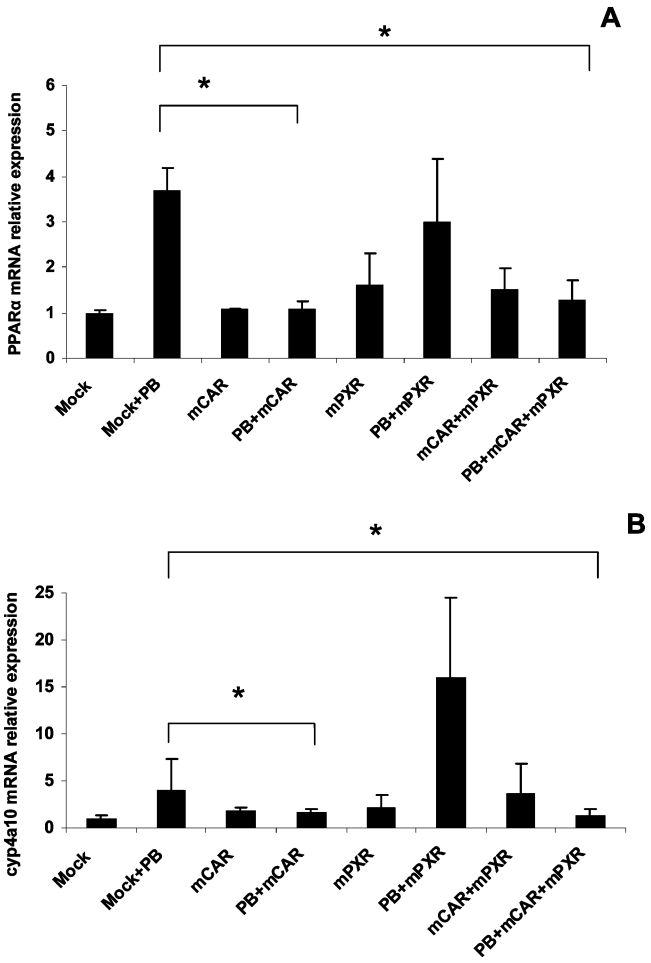

Figure 2. Effect of CAR and PXR on the induction of PPARα by phenobarbital. CAR/PXR$^{-/-}$ cells were transfected with expression constructs for mCAR and/or mPXR and treated with 1 mM PB. Total RNA was isolated as described and after reverse transcription the amount of cDNA was analyzed with RT-PCR. RT-PCR data are calculated relative to cyclophilin. (A) Relative expression of PPARα mRNA. (B) Relative expression of cyp4a10 mRNA (Mock: transfection with empty vector, $^*p < 0.05$).

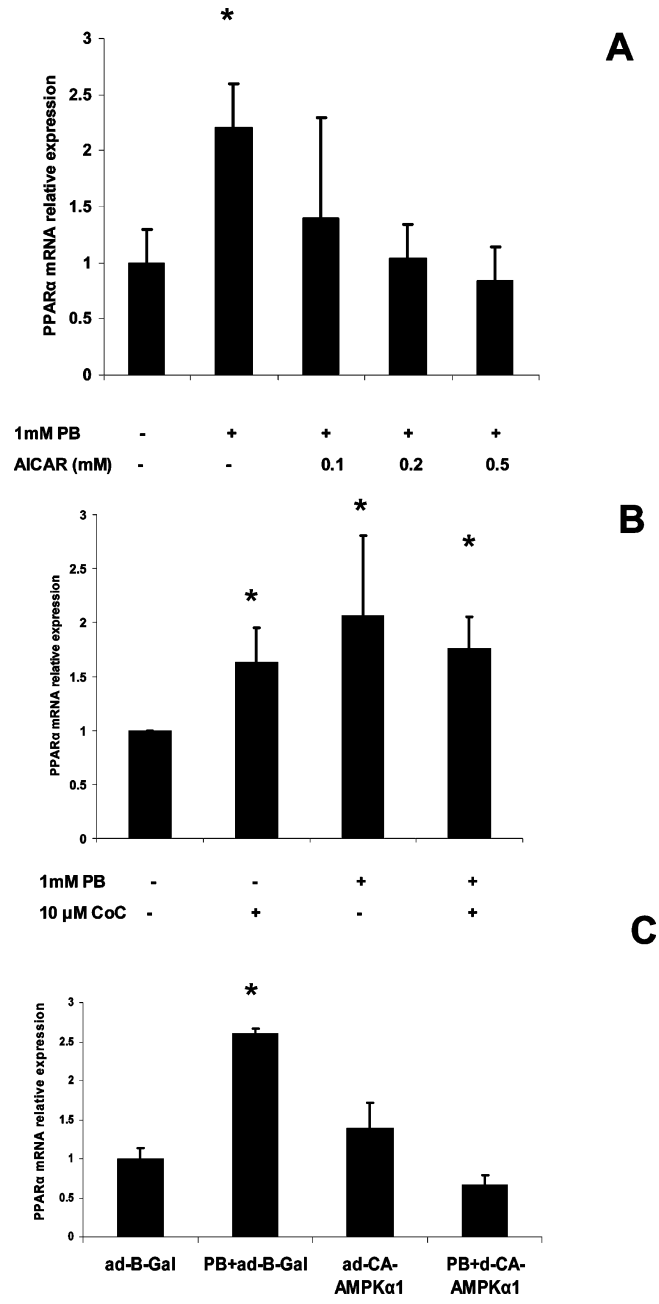

Figure 3. PPARα induction by PB can be reduced by activation of AMPK. Primary hepatocytes of CAR/PXR$^{-/-}$ mice were treated for 48 h with 1 mM PB and/or different concentrations of AICAR (0.1, 0.2, 0.5 mM) (A); 1 mM PB and/or 10 μM Comp C (B). Primary CAR/PXR$^{-/-}$ hepatocytes were transfected with empty plasmids (ad-B-Gal), or plasmids expressing constitutively active AMPKα1 (ad-CA-AMPKα1). Cells were treated with 1 mM PB for 48 h. Total RNA was analyzed with RT-PCR for PPARα levels (relative to cyclophilin, $^*p < 0.05$) (C).

(Figure 4B). Transfecting hepatocytes with HNF-4α expression plasmid produced an increase in PPARα mRNA, which was even higher in the presence of phenobarbital. By adding an increasing amount of CAR expression plasmid, the induction was prevented (Figure 4C). Transfection of cells with a plasmid expressing PXR suppressed the HNF-4α mediated transactivation (Figure 4D).

Discussion

In this paper we provide evidence for functional crosstalk between AMPK, CAR, PXR and HNF-4α in livers of mice exposed to phenobarbital. Gene expression studies in mice with targeted deficiencies of CAR and PXR suggested that under these conditions phenobarbital induces PPARα as well as some other nuclear receptors. Here we describe experiments which suggest a mechanism by which phenobarbital regulates PPARα and its target genes at the transcriptional level. This effect of phenobarbital was confirmed by quantitative PCR experiments in mice, showing that PB induces PPARα in the absence of PXR or CAR and CAR/PXR double-knockout mice (Table 2). CAR deletion also affected expression of Cyp4a10 but had no

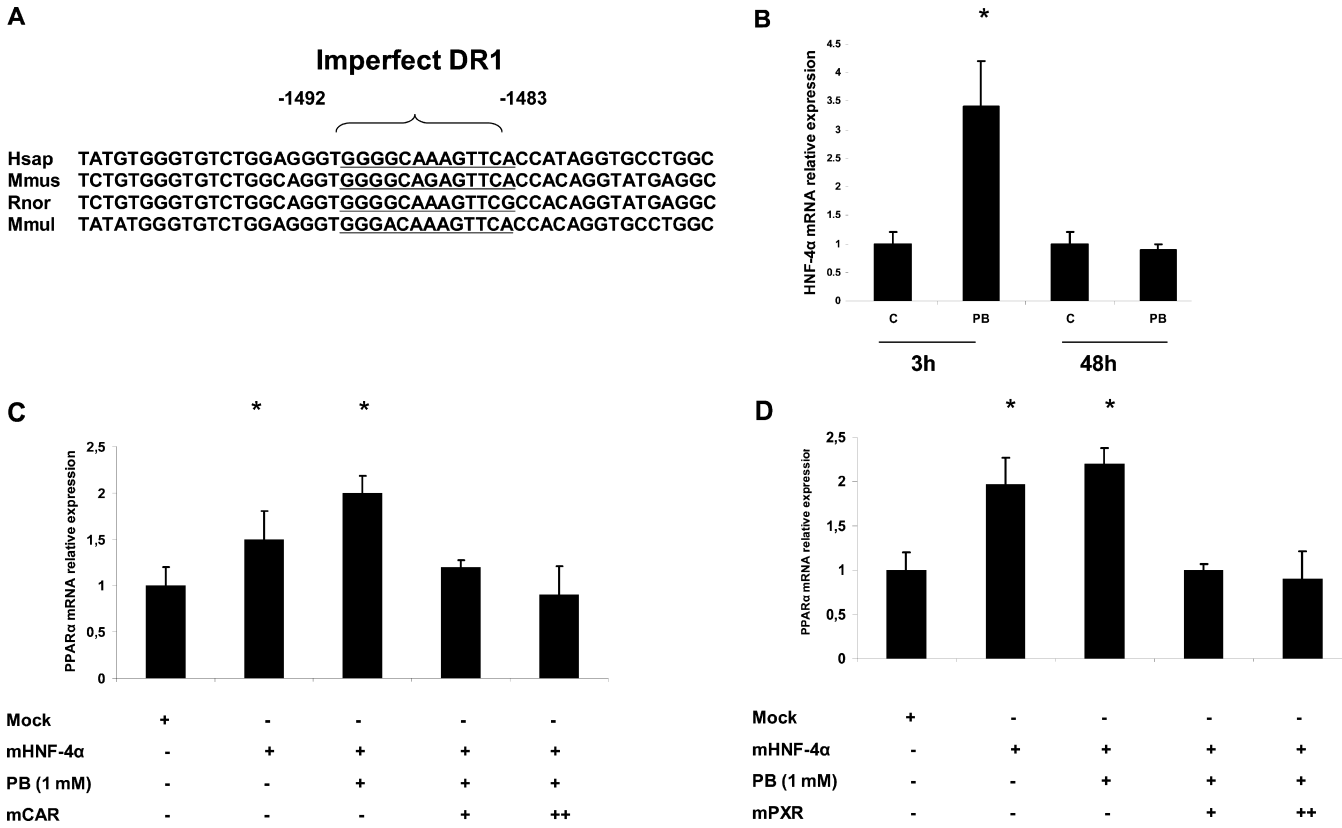

Figure 4. HNF-4α is involved in the induction of PPARα by phenobarbital in CAR/PXR$^{-/-}$ cells. Sequence comparison with ENSEMBL between different species for HNF-4 binding site in the 5′ flanking region of PPARα receptor. The human and mouse gene contains the same imperfect repeat between −1492 and −1483, except that the two half sites are divided by different nucleotide (hhum, *Homo sapiens*; mmus, *Mus musculus*; rnor, *Rattus norvegicus*; mmul, *Macaccus Mulatta*) (A). CAR/PXR$^{-/-}$ cells were induced with 1 mM PB for 3 h or 48 h and analyzed for HNF-4α mRNA level. (B) CAR/PXR$^{-/-}$ hepatocytes were transfected with empty plasmid (Mock), expression plasmid for mHNF-4α and two concentrations (marked as +, ++) of plasmid DNA for mCAR (C) or mPXR (D) and treated for 48 h with 1 mM PB. PPARα mRNA level was determinated by RT-PCR analysis (relative to cyclophilin, $^*p < 0.05$).

statistically significant effect on Cyp4a14, although it is known from the studies of Maglich et al. that TCPOBOP-activated CAR inhibits Cyp4A14 mRNA expression.[20] Ueda and coworkers reported that PB also induces Cyp4a10 and Cyp4a14 in the CAR$^{-/-}$ mice although the microarray data was validated just for Cyp4a10.[21] PXR deletion had no significant effect on neither Cyp4a10 nor Cyp4a14, mostly due to the large variability of the effects of PXR deletion. We therefore chose Cyp4a10 as PPARα target gene for further studies.

In the normal liver, phenobarbital activates AMPK and causes the transfer of CAR into the nucleus. Both effects are necessary for the induction of CAR-dependent genes such as Cyp2b10.[22,23] Transfection experiments show that the induction of PPARα depends on the absence of CAR and to a lesser extent of PXR (Figure 2). In the absence of CAR/PXR, phenobarbital presumably does not activate AMPK. We observed in preliminary experiments that there is less phosphoAMPK and phosphoACC protein after phenobarbital treatment in CAR$^{-/-}$ mice. Moreover, if AMPK is activated with another mechanism (e.g., by AICAR, see Figure 3A), or a constitutively active form of AMPK is transfected (Figure 3C), PPARα is not induced. AMPK, CAR and PXR have known effects on the transcriptional activation of HNF-4α. We therefore hypothesize that phenobarbital may regulate PPARα via HNF-4α (Figure 4). An induction of HNF-4α by phenobarbital in the absence of CAR and PXR indeed

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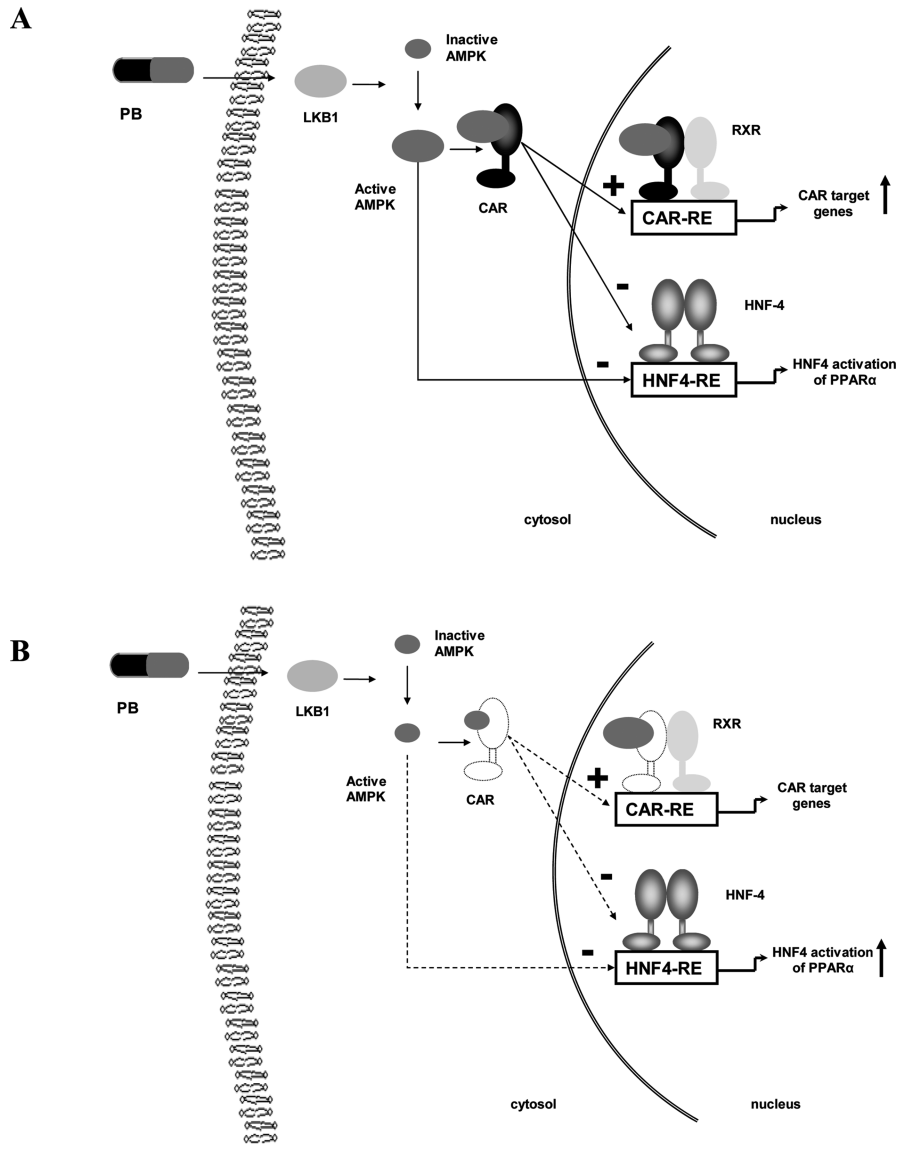

Figure 5. Schematic representation of PPARα induction by phenobarbital in wild type and CAR/PXR$^{-/-}$ animals. In wild type cells (A), phenobarbital activates LKB1, which further activates AMPK. Active AMPK coactivates CAR and decreases protein stability and dimerization of HNF-4α. Active CAR inhibits HNF-4α regulation by interacting with the receptor itself and at the same time it induces CAR target genes. In the CAR/PXR$^{-/-}$ deficient mice (B), PB will not activate CAR related genes, but it will activate HNF-4α target genes such as PPARα.

was previously observed by Bell and Michalopoulos.[24] Moreover, AMPK is known to phosphorylate HNF-4α and regulate its transcriptional activity by inhibiting dimer formation and protein stability.[25] Many studies prove that CAR and PXR interact with HNF-4α by interfering with HNF-4α binding to nuclear receptors and coactivators such as PGC1α.[26−30] Regulation of PPARα by HNF4α is consistent with the tissue expression pattern of PPARα,

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providing further evidence for the connection between these two nuclear receptors.[31] Finally, studies with HNF4 knockout mouse experiments showed that HNF-4α upregulates PPARα mRNA expression.[32,33]

In view of these observations and the present data, the following mechanism is proposed: inhibitory crosstalk between CAR, PXR and HNF-4α suppresses phenobarbital induction of PPARα in normal liver (Figure 4C,D). In the absence of CAR and in part of PXR, phenobarbital induces a different set of genes than in the wild type animals. There is no inhibitory crosstalk between CAR, PXR and HNF-4α, presumably no activation of AMPK, and therefore no destabilization of HNF-4α (Figure 5A,B). The combination of these three effects may result in the increased transcriptional activation by HNF-4α and the increase in PPARα. We also suggest that this may represent a general mechanism by which CAR and PXR downregulate gene expression in the liver.

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Abbreviations Used

ACC, acetyl-CoA carboxylase; AICAR, 5′-phosphoribosyl-5-aminoimidazole-4-carboximide; AMPK, AMP-activated kinase; CAR, constitutive androstane receptor (NR1I3); Comp C, compound C; CYP, cytochrome P450; CYCLO, cyclophilin; FXR, farnesoid X receptor (NR1h4); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GRIP-1, glutamate receptor-interacting protein; HNF-4α, hepatocyte nuclear factor 4, alpha (NR2a1); LXRβ, liver X receptor β (NR1h2); PB, phenobarbital; PGC-1α, PPAR gamma coactivator 1-alpha (PGC1α); PPARα, peroxisome proliferator-activated receptor alpha (NR1C1); PXR, pregnane X receptor (NR1I2); TCPOBOP, 3,3′,5,5′-tetrachloro-1,4-bis(pyridyloxy)benzene; PCN, 3β-hydroxy-20-oxopregn-5-ene-16α-carbonitrile.

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