Phenobarbital induces cytochrome P4501A2 hnRNA, mRNA and protein in the liver of C57BL/6J wild type and aryl hydrocarbon receptor knock-out mice

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Abstract The aryl hydrocarbon receptor mediates the transcriptional response to a variety of hydrocarbons of members of the aryl hydrocarbon gene battery. Phenobarbital does not bind the aryl hydrocarbon receptor with high affinity but induces, in liver cells, expression of cytochrome P4501A. Using both wild type and aryl hydrocarbon receptor knock out C57BL/6J mice, we demonstrate that phenobarbital induced hnRNA, mRNA and protein for the cytochrome P-4501A2 gene in the presence or absence of the aryl hydrocarbon receptor. Using the DNA binding site for the aryl hydrocarbon receptor as a probe, gel retardation analyses showed that phenobarbital treatment induced protein binding, regardless of the presence of the aryl hydrocarbon receptor.

Key words: Phenobarbital; Cytochrome P450; Aryl hydrocarbon receptor; Knock-out mouse; Liver

1. Introduction

The aryl hydrocarbon receptor (AHR) and the aryl hydrocarbon receptor nuclear translocator (ARNT) proteins belong to a small family of transcription factors (defining the PAS family) involved in DNA and/or ligand binding [1]. The well established role of the AHR is to transactivate genes in response to either xenobiotic compounds or other stimuli such as modification of cell shape and interactions with the extracellular matrix or other cells [2]. The DNA sequences recognized by the heteromeric AHR-ARNT complex have been named xenobiotic or drug response elements (XRE or DRE) and are generally present in several copies in the 5′-flanking region of responsive genes [3]. Following DNA binding, the activated receptor complex allows the inductive response through interaction with the basal transcription machinery [4,5].

Recently, the gene coding the AHR has been knocked out in mice by two separate groups to yield $AHR^{-/-}$ homozygous C57BL/6J mouse lines [6,7]. As expected, the inductive response of two of the genes positively regulated by the AHR, namely the cytochrome P4501A1 and 1A2 (CYP1A1 and CYP1A2) genes, to AHR ligands such as polycyclic aromatic hydrocarbons (PAH) (3-methylcholanthrene, 3-MC), was lost in $AHR^{-/-}$ mouse lines. However, expression of these genes has also been shown to be influenced by xenobiotics that are not high affinity ligands, or even not at all ligands of the receptor. Among these molecules, omeprazole, a gastric proton pump inhibitor, has recently been suggested to activate the AHR in the absence of direct binding, possibly through the generation of a metabolic derivative of very short half-life [8].

Another class of molecules, typified by the barbiturate phenobarbital (PB), has been shown, in several experimental systems, to activate expression of the CYP1A1 gene [9–13], although PB mainly induces transcription of genes that are different from those that respond to AHR ligands, including the rat CYP2B1 and 2B2 genes [14]. For instance, PB is able to induce a transcriptional activation of the CYP1A1 gene in trout hepatocytes and increased CYP1A1 mRNA accumulation in rat hepatocyte primary cultures or in rat hepatoma cells [10,15]. PB is also able to act in a synergistic fashion with 3-MC and benzanthracene, another AHR ligand, to induce the CYP1A1 gene, suggesting that the barbiturate and AHR ligands do not act through the same pathway [15,16]. In addition, both PAHs and PB can induce glutathione *S*-transferases of the alpha class (GSTYα), and the molecular events that underlie these responses are believed to be at least partly distinct [17].

The aim of the present study was to analyze the response to PB of CYP genes from subfamilies 1A, 2B and 3A, as well as GSTYα in mouse liver using both wild type (wt) and $AHR^{-/-}$ mice to directly address the question of the requirement for a functional AHR molecule in PB induction.

2. Materials and methods

2.1. Chemicals

PB sodium salt was from Coopération Pharmaceutique Française (Melun, France), 3-MC was from Sigma (St. Quentin Fallavier, France), poly(dI-dC) was from Pharmacia Biotech (St-Quentin-Yvelines, France). Acrylamide gel solution was from Appligene Oncor (Illkirch, France). The oligonucleotides were synthesized by Genset SA (Paris, France). M-MLV reverse transcriptase and DNase I were from Promega (Madison, WI, USA), and Taq DNA polymerase was from Life Technologies (Cergy Pontoise, France).

2.2. Animal housing and treatment

Mouse care was according to institutional guidelines. $AHR^{-/-}$ mice originate from homologous recombinant 129Sv-derived cells and were

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Abbreviations: AHR, aryl hydrocarbon receptor; $AHR^{-/-}$, knocked out AHR gene; Ah, aryl hydrocarbon; CYP, cytochrome P450; ARNT, aryl hydrocarbon receptor nuclear translocator; PAS, Per-ARNT-Sim; XRE, xenobiotic response element; DRE, drug response element; 3-MC, 3-methylcholanthrene; PB, phenobarbital; PAH, polycyclic aromatic hydrocarbon; wt, wild type; GSTYα, glutathione *S*-transferase alpha class; EDTA, ethylene diamine tetraacetic acid; TBE, Tris-borate-EDTA

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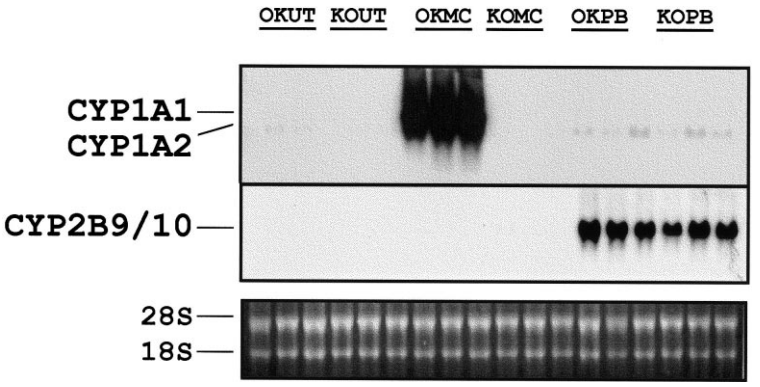

Fig. 1. RNA blot analysis of the CYP1A1, CYP1A2 and CYP2B9/10 genes in wt and AHR$^{-/-}$ mice. 10 μg of total RNA from three animals from the same treatment group was resolved on denaturing gels. Following transfer to nylon membranes, hybridization experiments were performed as described in Section 2. OKUT, OKMC and OKPB stand for untreated, 3-MC-treated and PB-treated wt animals, respectively. KOUT, KOMC and KOPB stand for untreated, 3-MC-treated and PB-treated AHR$^{-/-}$ animals, respectively. A low and a very low basal level were detected with the CYP2B9/10 and CYP1A2 cDNA probes, respectively, on longer exposure of RNA filters.

derived in collaboration with one of us [6]. Chimeric males were initially backcrossed to C57BL/6J females. For the purpose of this study, several additional rounds of backcrossing were performed in our animal facility to increase the C57BL/6J genetic background in heterozygous breeders and generate the homozygous mutant animals used. Wt C57BL/6J used as controls were from IFFA-CREDO (Les Oncins, France). Mice were housed under conventional conditions in individual cages. Animal sampling was designed to reduce the influence of interfering factors (parental imprinting, litter specificity). Mice received a regular chow diet. Water and food were available ad libitum.

2.3. RNA extraction and hybridization

Total RNA was isolated according to [18] from fresh liver biopsies homogenized in Trizol solution (Life Technologies, Cergy Pontoise, France) using a tissue homogenizer (Ika, Jena, Germany) with a 7 mm diameter probe. DNase I-treated hnRNAs for the CYP1A2 gene were analyzed by semi-quantitative reverse transcriptase (RT)-PCR using primers from intron B (forward primer: 5′-TGGGGTTATGG-GAAAGAAGGG-3′) and intron C (reverse primer: 5′-CACACCTT-GATCTTAGGGCAGG-3′), as described [19]. PCR products were separated on agarose gels, transferred onto nylon membranes (Hybond N$^+$, Amersham, Evry, France) and hybridized with a mouse CYP1A2 probe. Cytoplasmic RNAs were resolved on denaturing gels, transferred to Hybond N membranes (Amersham, Evry, France) and hybridized as described [20] sequentially to full-length cDNA probes for the mouse CYP1A2, the mouse CYP2B9, the rabbit CYP3A, the mouse GSTYα, the mouse GAPDH and an oligonucleotide probe for the 18S ribosomal RNA. Hybridization signals were standardized to that of GAPDH which was unaffected by the various treatment conditions, by using the Densylab imaging software (Microvision Instruments, Evry, France). Equivalent RNA loading and integrity were demonstrated by ethidium bromide staining of the gels and hybridization to the 18S oligonucleotide probe.

2.4. Western blot analysis

Liver microsomes were prepared according to standard procedures. Proteins in microsomal samples were quantified using the reagent from Pierce (Rockford, IL, USA). 8 μg of protein was loaded in each lane and separated in 7.5% polyacrylamide gels. Samples were electrophoretically blotted onto nitrocellulose membranes (Optitran BA-S 85, Schleicher and Schuell, Dassel, Germany) before saturation with milk proteins. Primary rabbit antibodies against CYP1A1 and CYP1A2 (a gift from Dr. F.J. Gonzalez, National Institute of Health, Bethesda, MA, YSA) were revealed using a peroxidase-conjugated antibody (Sigma, St. Quentin Fallavier, France) and a chemiluminescent reagent (Pierce, Rockford, IL, USA) following autoradiography.

2.5. Gel retardation assays

Nuclear extracts were isolated as described [21]. Binding reactions using 4 μg of nuclear protein samples were carried out in a 15 μl volume containing 1 mM sodium phosphate (pH 7.5), 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% (v/v) glycerol, 2 μg poly(dI-dC), 1 mM $MgCl_2$, 10 mM spermidine, 0.1–0.2 ng of ^{32}P-labeled 5′-end oligonucleotide. The XRE (DRE3) oligonucleotide probe had the following sequence: 5′-GATCTGGCTCTTCTCAC-3′. 4 μg nuclear protein was added to the reaction mixture and incubated for 30 min at 24°C. The DNA-protein complexes were resolved on 6% acrylamide gels in 0.5×TBE (45 mM Tris-borate, 1.25 mM EDTA) at 22 mAmp for 2 h. The gels were then fixed, dried, and subjected to autoradiography.

3. Results and discussion

3.1. PB induces CYP1A2 mRNA in the liver of both wt and AHR$^{-/-}$ mice

Male mice from the C57BL/6J strain were treated for 12 h with a single intraperitoneal injection of either sodium PB

Table 1
Induction factors for CYP1A1, CYP1A2, CYP2B9/10, CYP3A11/13 and GSTYα mRNA levels in wt and AHR$^{-/-}$ mice

	OKUT	KOUT	OKMC	KOMC	OKPB	KOPB
CYP1A1[a]	na	na	+++	na	na	na
CYP1A2	1 ± 0.2	< 1	20 ± 3	< 1	3 ± 1	> 5 ± 2
CYP2B9/10	1 ± 0.1	1 ± 0.1	1 ± 0.2	1 ± 0.1	22 ± 2	21 ± 3
CYP3A11/13	1 ± 0.1	1 ± 0.2	1 ± 0.2	1 ± 0.1	17 ± 3	17 ± 2
GSTYα	1 ± 0.3	1 ± 0.2	3 ± 0.2	1 ± 0.3	5 ± 2	5 ± 1

The induction factors represent the mean ± standard deviation from three separate RNA samples under each condition, and were determined by comparison with the respective control (i.e. OKPB and OKMC versus OKUT; KOPB and KOMC versus KOUT). OKUT, OKMC and OKPB stand for untreated, 3-MC-treated and PB-treated wt animals, respectively. KOUT, KOMC and KOPB stand for untreated, 3-MC-treated and PB-treated AHR$^{-/-}$ animals, respectively. For a given probe, basal mRNA levels were essentially identical between all individuals, except for the CYP1A2 probe which showed a strongly decreased basal level in AHR$^{-/-}$ mice as compared to wt mice.

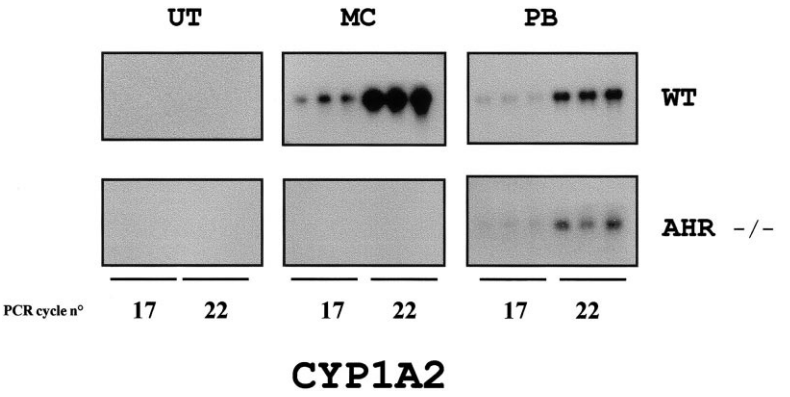

Fig. 2. CYP1A2 hnRNA analysis in wt and AHR$^{-/-}$ mice. Reverse transcribed hnRNAs (corresponding to 125 ng of total RNA) from three animals from the same treatment group were subjected to PCR amplification for various cycle numbers in a 50 μl volume. 5 μl was separated on a 0.8% agarose gel, transferred onto nylon membranes and hybridized with a CYP1A2 cDNA probe. UT, PB and MC stand for untreated, 3-MC-treated and PB-treated animals, respectively. The expected 435 bp CYP1A2-derived cDNA fragment [19] was visualized following autoradiography.

(100 mg/kg) or 3-MC (80 mg/kg) and the livers were promptly removed and frozen or used to prepare total RNA 12 h later. As shown in Fig. 1, PB readily induced CYP1A2 mRNA (2 kbp long), but not CYP1A1 (3 kbp long), in both wt and AHR$^{-/-}$ mice, despite the lower basal level of this message in AHR$^{-/-}$ mice. We have made similar observations at later time points and a lower PB dose (3 days and 50 mg/kg, data not shown). However, 3-MC had no detectable effect in AHR$^{-/-}$ mice, although it induced both CYP1A1 and CYP1A2 mRNAs in wt mice, as expected. In addition, PB induction of known PB-responsive genes was unaffected in the AHR$^{-/-}$ animals (Table 1), in agreement with the notion that a functional AHR is not required for the PB response of either CYP2B or CYP3A genes in the liver [14]. In addition, the 3-MC induction, but not the PB induction of the GSTYα genes was abolished in AHR$^{-/-}$ mice.

These results unambiguously demonstrate that PB induction of the CYP1A2 and the GSTYα genes in mice does not require a functional AHR. In agreement with these observations, it was recently reported that piperonyl butoxide and acenaphthylene induce CYP1A2 mRNA in AHR$^{-/-}$ animals from the same origin [19].

3.2. *PB induces CYP1A2 hnRNA in both wt and AHR$^{-/-}$ mice*

A survey of hnRNA levels by specific RT-PCR using a combination of primers from two introns has been proposed as a substitute to nuclear run on assays. Although the method does not determine the actual level of newly synthesized mRNA, it reflects both transcriptional and post-transcriptional processing, but not mRNA stabilization or destabilization like RNA blotting experiments would [22]. The method applied to the mouse CYP1A2 gene has been published previously [19]. Using this strategy, we demonstrated that both PB and 3-MC strongly induced CYP1A2 nuclear RNA in wt mice, but only PB also induced this primary transcript in AHR$^{-/-}$ animals (Fig. 2). Control experiments using specific CYP1A1 primer pairs showed that 3-MC, but not PB, strongly induced CYP1A1 hnRNA, in agreement with the results from RNA blotting experiments (see above), and this only in wt mice (data not shown).

3.3. *The CYP1A2 protein is induced by PB in both wt and AHR$^{-/-}$ mice*

Microsomal protein samples from the same animals as used above were prepared and analyzed as described in Section 2. The results presented in Fig. 3 show that only the CYP1A2 protein was detectable under basal conditions, and was strongly reduced in AHR$^{-/-}$ mice, as expected [6]. 3-MC induced both the CYP1A1 and the CYP1A2 proteins in wt animals, but had no effect in AHR$^{-/-}$ mice. However, PB induced the CYP1A2, but not the CYP1A1 protein, in both wt and AHR$^{-/-}$ mice. The PB-induced protein level was somewhat lower than that obtained in wt mice, most probably because of a decreased basal level. It could also be that there is a partial requirement for the AHR. These results show that PB is able to induce the CYP1A2 protein in the liver in both the presence and the absence of the AHR, in agreement with its effects on CYP1A2 mRNA levels.

3.4. *AHR gene disruption does not prevent protein binding to the XRE*

Nuclear protein extracts were used to look at protein binding to one XRE element in either wt or AHR$^{-/-}$ mice treated or not with PB or 3-MC. Our results show that a specific DNA binding activity could be detected in wt animals treated

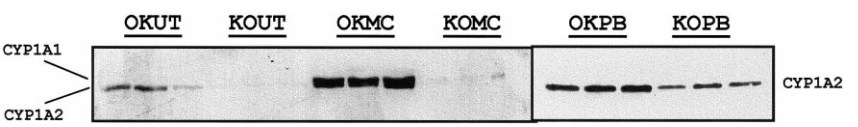

Fig. 3. Western blot analysis of the CYP1A1 and CYP1A2 proteins in wt and AHR$^{-/-}$ mice. 8 μg of microsomal proteins from three animals from the same treatment group was resolved on denaturing acrylamide gels. Following transfer to nitrocellulose membranes, antibody recognition experiments were performed as described in Section 2.

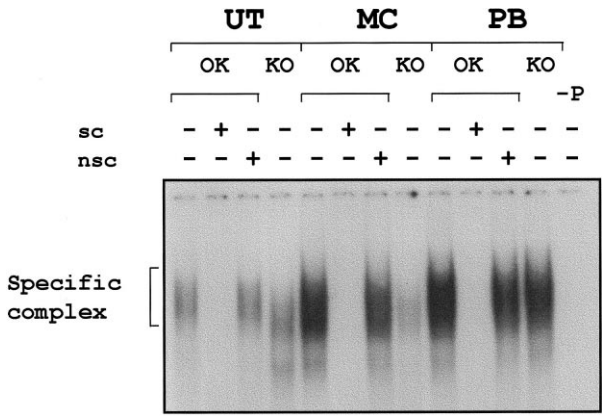

Fig. 4. Gel retardation assay with nuclear proteins from wt and $AHR^{-/-}$ mice. 4 μg of nuclear protein extracts was resolved on native acrylamide gels, as described in Section 2. Pooled samples from three animals from the same treatment group were used. Inter-individual variability was estimated to be less than 15% in pilot experiments. DNA binding specificity was ascertained by using both specific (sc) and non-specific (nsc) (AP1) competitors in a 50-fold excess. OKUT, OKMC and OKPB stand for untreated, 3-MC-treated and PB-treated wt animals, respectively. KOUT, KOMC and KOPB stand for untreated, 3-MC-treated and PB-treated $AHR^{-/-}$ animals, respectively.

or not with 3-MC or PB (Fig. 4). However, a much lower DNA binding activity was detected in $AHR^{-/-}$ mice untreated or treated with 3-MC. This observation could explain, at least partly, the decreased constitutive expression level of the CYP1A2 mRNA in $AHR^{-/-}$ animals and the lack of responsiveness to 3-MC of both the CYP1A1 and 1A2 genes in these animals. Strikingly, a strong binding activity was observed following PB treatment of wt mice and remained similar in intensity in $AHR^{-/-}$ mice, suggesting that the major proteins binding to the XRE in response to PB are distinct from the AHR. Therefore, it appears that the AHR is indeed able to bind to the XRE sequence, but, in addition, in its absence, a protein(s) can interact with this motif as a result of PB treatment. One or more of the recently described new PAS proteins could be involved in this DNA recognition potential in mice [23].

Although indicative of a possible role of PB through the XRE motif, these results do not provide any clue as to whether PB acts through this DNA sequence element to induce expression of the CYP1A2 gene. In addition, transfection experiments of a construct containing two copies of the XRE sequence driving expression of the chloramphenicol acetyltransferase gene failed to demonstrate PB-inducible expression in primary hepatocyte cultures (data not shown). This absence of detectable PB responsiveness is most likely due to the low sensitivity of the assay. Alternatively, it is possible that the presumptive XRE motifs from the CYP1A promoter region cannot be efficiently transactivated by PB when removed from the context of the native promoter. Furthermore, 1843 bp of the immediately upstream region of the mouse CYP1A2 gene failed to demonstrate the presence of functional AHR-ligand responsive sequences upon transfection of mouse hepatoma cells [24]. This observation suggests that the DNA sequences present in this portion of the CYP1A2 gene locus either do not contain functional XRE sequences or lack other structural determinants. Interestingly, it has also been proposed that induction of the human CYP1A2 gene by 3-MC might require both a XRE-like sequence centered around position −2496 bp and an AP1 binding site located at −2031 bp upstream of the transcriptional start site [25]. In this respect, AP1-related proteins have been suggested to play a positive role in the PB induction of the rat CYP2B2 and mouse GSTYα genes in view of the ability of the barbiturate to induce increased band shift of nuclear proteins with the AP1 DNA binding site, and to stimulate transcription in transient transfection assays with the GSTYα promoter [17].

In conclusion, our results show that PB is able to increase the levels of CYP1A2 – but not CYP1A1 – hnRNA, mRNA and protein in C57BL/6J mice irrespective of the presence of a functional AHR. Although it is formally possible that this receptor could be required, directly or indirectly, for the PB response in wt but not in $AHR^{-/-}$ mice, the simplest hypothesis would certainly argue that PB induction of the CYP1A2 gene does not require the AHR.

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