

The Orphan Nuclear Receptor Constitutive Active/Androstane Receptor Is Essential for Liver Tumor Promotion by Phenobarbital in Mice

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Abstract

Hepatocellular carcinoma (HCC) is known to progress through a step often called tumor promotion. Phenobarbital (PB) is the prototype of nongenotoxic carcinogens that promote HCC in rodents. The molecular target of PB to elicit the promotion has been the subject of intense investigations over the last 30 years since it was discovered. The nuclear receptor constitutive active/androstane receptor (CAR) is activated by PB as well as by various other xenobiotics such as therapeutic drugs and environmental pollutants. CAR activation results in the transcriptional induction of numerous hepatic genes including those that encode xenobiotic-metabolizing enzymes such as a set of cytochrome P450s. In addition to PB, many CAR activators are nongenotoxic carcinogens, but the role of CAR in liver tumor promotion remains unexplored. Using *Car*^{-/-} mice, we have here examined tumor promotion by chronic treatment with PB in drinking water after tumor initiation with a single dose of the genotoxic carcinogen diethylnitrosamine. None of the *Car*^{-/-} mice developed either eosinophilic foci or advanced liver tumors, whereas all *Car*^{+/+} mice developed HCC and/or adenoma by 39 weeks. The results indicate that CAR is the molecular target of promotion by PB and that activation of this receptor is an essential requirement for liver tumor development.

Introduction

Hepatocellular carcinoma (HCC) comprises 90% of liver tumors occurring worldwide in humans, with more than half a million new cases reported every year. Diagnosis of this tumor, once restricted mostly to Asia, is now rising in Europe as well as North America (1). Environmental factors, such as viral hepatitis and chemical exposure, are causally implicated in HCC occurrence and development of HCC is a multistage process, involving initiation, promotion and progression (2). The molecular-based mechanism(s) underlying each stage of HCC development remains enigmatic. In rodents, HCC can be produced by initiating with genotoxic carcinogens (e.g., nitrosamines) and subsequent promotion by nongenotoxic agents such as phenobarbital (PB), 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, and 17 α -ethynylestradiol (3–5). Such rodent models have been used to provide insight into understanding HCC development and, in particular, the liver tumor promotion that occurs without gene mutation.

Development of eosinophilic foci and adenoma in rodents is characteristic of liver tumor promotion by agents such as PB (6). The mutation of the β -catenin gene, which occurs frequently in human HCC, has been observed frequently in the PB-promoted eosinophilic foci (7, 8). It was shown that in mice lacking the *c-Jun* gene, early

development of liver tumors by PB was reduced (9). In addition, knocking out genes such as *Connexin32* and *N-acetylglucosaminyltransferase III* has also been reported to reduce the PB-induced tumor promotion in these mice (10, 11). Despite the apparent correlation of tumor promotion with these genes, it has not been shown that they are direct targets of PB action. Typified by the prototypic PB, nongenotoxic carcinogens generally induce hepatic xenobiotic-metabolizing enzymes such as cytochrome P450 (CYP) and certain transferases. Moreover, enzyme induction is tightly correlated with HCC development (12), although an endogenous factor directly connecting them has not yet been identified. The nuclear receptor constitutive active/androstane receptor (CAR) was first identified as a PB-activated transcription factor that regulates the genes encoding those enzymes (13–15). We have now used *Car*^{-/-} mice to investigate the role of CAR in the development of HCC and present the experimental evidence indicating an essential role for this receptor in liver tumor promotion by PB.

Materials and Methods

Animals. The original background strain of *Car*^{+/+} and *Car*^{-/-} mice used in our previous work (16) was changed to a liver tumor-susceptible mouse strain by repeated backcrossing to C3H/HeNcr1BR mice (Charles River Laboratories, Wilmington, MA) until microsatellite analysis showed that mice contained 98% of the C3H markers. Mice were housed in a specific pathogen-free rodent facility, and experiments were done in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. The genotypes of offspring were determined by analyzing the mutant allele using polymerase chain reaction (PCR) with genomic DNA.

Hepatocarcinogenesis Protocol and Analysis of Mice. Tumor initiation and promotion were carried out according to a standard protocol (17). A total of 269 male mice were given a single intraperitoneal injection of diethylnitrosamine [DEN (90 mg/kg)] at 5 weeks of age and divided into four groups (group 1, 70 *Car*^{+/+} mice; group 2, 71 *Car*^{+/+} mice; group 3, 62 *Car*^{-/-} mice; and group 4, 66 *Car*^{-/-} mice). The mice in groups 2 and 4 were chronically treated with PB (500 ppm) in drinking water at 7 weeks of age until they were sacrificed or moribund. Seventy-two and 79 mice were randomly selected and underwent necropsy at 30 and 39 weeks of age, respectively. The remaining 118 mice were kept for further investigations including an analysis of survival. Sera were collected, and the livers were weighed and saved for analysis. Macroscopic liver lesions were visually counted. Liver sections were stained with hematoxylin and eosin and evaluated blindly for hepatocellular proliferative lesions according to established criteria (18). Bromodeoxyuridine (BrdUrd) was administered in the drinking water (200 ppm) for 3 days before necropsy. Immunohistochemical staining was performed on 10% formalin-fixed, paraffin-embedded sections. The mouse monoclonal anti-BrdUrd and anti- β -catenin antibodies (Transduction Laboratories, Lexington, KY) were used, respectively. Immunoreactivity was visualized with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine (Dako, Carpinteria, CA). Slides were counterstained with hematoxylin.

Food and Water Consumption. Mice received Purina Pico Chow #5058 (Ralston Purina Co., St. Louis, MO) and water *ad libitum*. Food consumption was measured over 7 days and calculated on a gram of feed consumed/body weight basis and showed no statistically significant difference between *Car* genotypes or treatments. In addition, no difference in water consumption was

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observed between $Car^{+/+}$ and $Car^{-/-}$ mice, but water consumption was slightly greater in PB-treated groups.

RNA Isolation and Real-Time Polymerase Chain Reaction. TRIzol reagent (Invitrogen, Carlsbad, CA) was used to isolate total RNA from the livers of mice treated with PB for 23 weeks. RNA was reverse transcribed by Superscript II (Invitrogen) to synthesize cDNA. Real-time PCR was carried out with an ABI Prism 7700 (Applied Biosystems, Foster City, CA), using appropriate TaqMan probe and primers 5'-6FAM-CATCACGGCCAATGTTATCTGCTCCA-TAMRA-3', 5'-ACCCACGTTCTCTTCCA-3', and 5'-CAGCAGGCGCAAGAACTGA-3' for CYP2B10. Assay-on-Demand Gene Expression Products (Applied Biosystems) were used as probe for NADPH-CYP reductase (Mm00435876-ml) and CYP3A11 (Mm00731567-ml). CYP2B10, CYP3A11, and reductase mRNAs were quantified by normalizing them against glyceraldehyde-3-phosphate dehydrogenase (TaqMan rodent glyceraldehyde-3-phosphate dehydrogenase control reagent; Applied Biosystems).

Results and Discussion

C57BL/6 mice are resistant to the development of liver tumors, and tumor occurrence is less than one-tenth that of C3H/He mice (17). To begin our tumor study, the C57BL/6X129 background of the original $Car^{-/-}$ mice (16) was first changed to C3H/He by repeated backcrosses. The $Car^{-/-}$ C3H/He mice were viable, and newborns appeared normal and healthy. Groups of $Car^{+/+}$ and $Car^{-/-}$ mice received injection with DEN to initiate liver tumors, and half of the mice in each group were subsequently treated with PB in drinking water. Twenty mice from each group were subjected to a pilot necropsy to examine induction of hepatic genes and assess tumor development after 23 weeks of PB treatment. First, real-time PCR was performed to evaluate the expression of known CAR target genes, *Cyp2b10*, *Cyp3a11* and *NADPH-CYP reductase* (CYP Red; Fig. 1A). CYP2B10 mRNA was strongly induced in all DEN+PB-treated

$Car^{+/+}$ mice (>100-fold induction compared with that in the DEN-treated $Car^{+/+}$ mice). No induction of the mRNA was observed in any of the DEN+PB-treated $Car^{-/-}$ mice. Western blots also showed the absence of CYP2B10 protein in the liver of those $Car^{-/-}$ mice (data not shown). CYP reductase transfers electrons from NADPH to CYP and is an essential component of the CYP-mediated monooxygenase system. CYP reductase mRNA was also induced only in the $Car^{+/+}$ mice. In addition, PB treatment preferentially increased CYP3A11 mRNA in the $Car^{+/+}$ mice; the average inductions were 3.0- and 1.25-fold in the $Car^{+/+}$ and $Car^{-/-}$ mice, respectively. The results showed clearly that PB, even with chronic treatment, does not induce these genes in the absence of CAR. Proliferative liver lesions had already developed in the DEN+PB-treated $Car^{+/+}$ mice by 23 weeks of PB treatment (Table 1). The DEN+PB-treated $Car^{+/+}$ mice exhibited a 16-fold higher multiplicity of proliferative liver lesions than the DEN+PB-treated $Car^{-/-}$ mice. Liver weight as well as serum alanine aminotransferase (ALT) and alkaline phosphatase (ALP), indicative of liver damage during tumor development, also began to increase only in DEN+PB-treated $Car^{+/+}$ mice (Table 1). However, in the $Car^{-/-}$ mice, only small putative preneoplastic foci of cellular alteration were found and no histologically diagnosed liver neoplasia was observed. At 32 weeks of PB treatment, all DEN+PB-treated $Car^{+/+}$, but none of the $Car^{-/-}$ mice, developed advanced liver tumors (Fig. 1B). As expected, liver weight and ALT and ALP were profoundly elevated in the DEN+PB-treated $Car^{+/+}$ mice at this time point, whereas such elevations were observed neither in the other treated mice (Table 1) nor in the respective untreated mice (data not shown). Thorough visual analysis of the livers revealed large lesions and high multiplicity in all DEN+PB-treated $Car^{+/+}$ mice (Table 1). In sharp contrast, the DEN+PB-treated $Car^{-/-}$ mice had

Fig. 1. Lack of advanced liver tumor in $Car^{+/+}$ mice. A, induction of drug-metabolizing enzymes. Real-time PCR was performed separately to measure expression levels of CYP2B10, NADPH-CYP reductase (CYP Red), and CYP3A11 mRNAs in the liver of each mouse. Each sample was performed in triplicate. B, representative livers from mice at 32 weeks after PB promotion. The liver from a DEN+PB-treated $Car^{+/+}$ mouse has multiple confluent tumors. Scale bar = 1 cm. C, histologic features of liver tumors. An eosinophilic adenoma protruding above the natural surface of the liver in a DEN+PB-treated $Car^{+/+}$ mouse. The liver section was stained with hematoxylin and eosin (a). BrdUrd staining in an eosinophilic adenoma (right half of figure) compared with the normal hepatic parenchyma (b). Immunohistochemistry showing nuclear localization of β -catenin (top of figure) in an eosinophilic adenoma (c). A hematoxylin and eosin-stained normal section from the liver of a DEN+PB-treated $Car^{-/-}$ mouse (d).

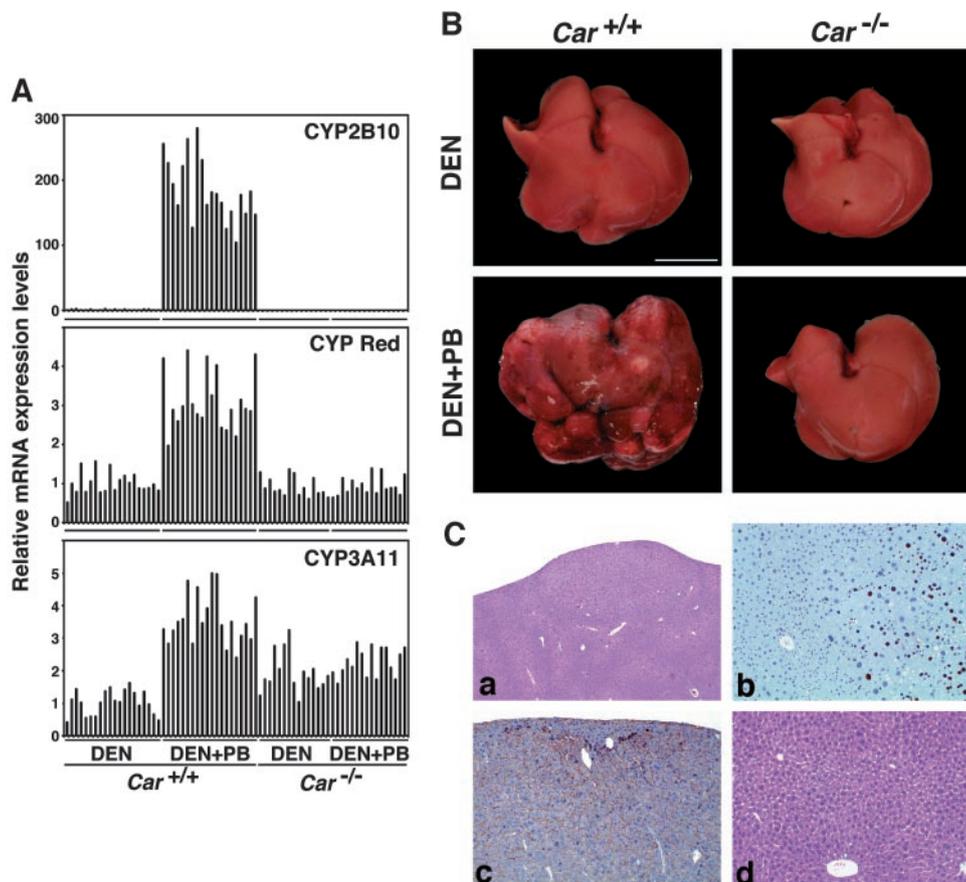


Table 1 Proliferative liver lesions, relative liver weight, and serum enzymes

Genotype	Treatment	n	Multiplicity*			Liver weight (%)‡	ALT§	ALP§	
			Total	≤2†	3-4				≥5
23 weeks of PB treatment									
<i>Car</i> ^{+/+}	DEN	20	0.9 ± 0.2	0.8	0.1	0	4.49 ± 0.05	35 ± 5	69 ± 2
<i>Car</i> ^{+/+}	DEN+PB	20	6.4 ± 1.8	5.6	0.6	0.2	5.89 ± 0.07	48 ± 4	87 ± 3
<i>Car</i> ^{-/-}	DEN	15	1.2 ± 0.3	1.1	0.1	0	4.28 ± 0.04	28 ± 2	81 ± 3
<i>Car</i> ^{-/-}	DEN+PB	17	0.4 ± 0.2	0.4	0	0	4.76 ± 0.08	30 ± 4	82 ± 4
32 weeks of PB treatment									
<i>Car</i> ^{+/+}	DEN	20	3.7 ± 0.6	3.2	0.4	0.2	4.52 ± 0.12	27 ± 2	73 ± 11
<i>Car</i> ^{+/+}	DEN+PB	20	36.4 ± 4.2	27.7	5.5	3.2	8.57 ± 0.72	133 ± 21	161 ± 21
<i>Car</i> ^{-/-}	DEN	20	3.4 ± 0.8	3.1	0.2	0.1	4.33 ± 0.08	27 ± 2	63 ± 2
<i>Car</i> ^{-/-}	DEN+PB	19	2.4 ± 0.7	2.3	0.1	0	4.32 ± 0.08	28 ± 2	68 ± 2

* Grossly observed proliferative lesions/mouse ± SE.

† Lesion size (in mm).

‡ Relative to body weight ± SE.

§ Units/liter ± SE.

Table 2 Incidences of proliferative liver lesions after 32 weeks of PB treatment

Genotype	Treatment	n	Carcinoma/adenoma	Carcinoma	Eosinophilic adenoma	Other adenoma*
<i>Car</i> ^{+/+}	DEN	20	4/20	1/20	0/20	4/20
<i>Car</i> ^{+/+}	DEN+PB	20	20/20	8/20	19/20	8/20
<i>Car</i> ^{-/-}	DEN	20	3/20	0/20	0/20	3/20
<i>Car</i> ^{-/-}	DEN+PB	19	0/19	0/19	0/19	0/19

* Basophilic, mixed, clear cell, or amphophilic adenoma.

approximately 15-fold fewer liver lesions that were on average less than 2 mm. The phenotypes of the lesions in these *Car*^{-/-} mice were similar to those in the DEN-treated *Car*^{+/+} and *Car*^{-/-} mice. Histologic analysis revealed eosinophilic adenoma, increased BrdUrd staining, and nuclear localization of β -catenin in the DEN+PB-treated *Car*^{+/+} mice (Fig. 1C). The nuclear localization of β -catenin is a suggested characteristic of eosinophilic liver lesions (7). The eosinophilic adenomas were found in 19 of 20 DEN+PB-treated *Car*^{+/+} mice, but in none of the DEN+PB-treated *Car*^{-/-} mice. With respect to the hepatocellular adenoma and carcinoma (Table 2), all DEN+PB-treated *Car*^{+/+} mice, but none of the DEN+PB-treated *Car*^{-/-} mice, developed either or both of them; this difference was statistically significant ($P < 0.001$) by Fisher's exact test. These results strikingly indicated a lack of liver tumor promotion by PB in the *Car*^{-/-} mice, which was further substantiated by the survival of the mice. The DEN+PB-treated *Car*^{+/+} mice started to die at 39 weeks, and all of them died by 52 weeks of PB treatment (Fig. 2). All of the dead mice bore multiple and large liver tumors.

In the absence of administration of specific promoter, DEN-initiated liver neoplasia is assumed to occur based on endogenous promotion and typically results in the development of basophilic and other noneosinophilic tumor phenotypes. Noneosinophilic adenomas were observed in the DEN-treated *Car*^{+/+} (4 of 20) and *Car*^{-/-} (3 of 20) and DEN+PB-treated *Car*^{+/+} (8 of 20) mice (see "Other adenoma" in Table 2), with no significant differences in incidence. However, the DEN+PB-treated *Car*^{-/-} mice did not develop adenoma of any phenotype, which was statistically different from that observed in the DEN+PB-treated *Car*^{+/+} mice ($P < 0.001$). Thus, PB treatment appeared to inhibit DEN-initiated tumor formation when complemented with the *Car*^{-/-} genotype. A tumor-preventive effect of PB was reported previously in which DEN was injected into preweaned B6C3F1 mice, and the subsequent PB treatment decreased the number of liver tumors (19). Whether the tumor-inhibitory effect of PB observed with the preweaned mice is mechanistically similar to that observed with the *Car*^{-/-} mice remains an interesting question for future research.

Our present study provided direct evidence that CAR activation is essential in liver tumor promotion by PB. A recent DNA microarray analysis of hepatic gene expression in *Car*^{-/-} mice showed that CAR

up- and down-regulates more than 70 genes. In addition to the coordinated up-regulation of drug-metabolizing enzymes, CAR down-regulated genes that encode gluconeogenic enzymes, those involved in fatty acid oxidation, and proteins such as angiogenin and fibronectin (16). Nongenotoxic carcinogens such as PB are known to stimulate cell proliferation and suppress apoptosis, leading to tumor development. Because the signaling mechanisms that regulate energy metabolism are interconnected with those controlling cell proliferation and apoptosis, the role of CAR might inadvertently extend to alter the fate of initiated cells. Our preliminary examination found that the expression of the oncogenes *c-Jun* and *c-Myc* was down-regulated and that expression of the *Mdm2* gene was slightly elevated in the *Car*^{-/-} mice, yet those changes displayed no obvious correlation with PB promotion (data not shown). The microarray study also revealed that more than 70 PB-modulated genes are not regulated by CAR (16). Whereas the possibility cannot be ruled out that, in addition to CAR,

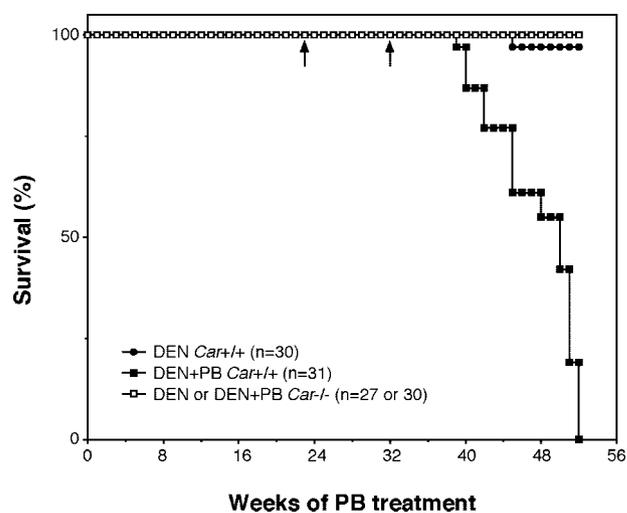


Fig. 2. Survival curves of *Car*^{+/+} and *Car*^{-/-} mice. PB treatment was continued until mice were humanely sacrificed when moribund, based on but not limited to the conditions of mice with respect to their breathing difficulty, rapid growing internal mass(es), and severe dehydration. Arrows indicate the weeks of necropsy.

another PB-regulated factor or factors may equally be required for the development of PB-promoted liver neoplasia, identifying the downstream event of CAR activation that actually leads to liver tumor development is an urgent issue of our current investigations.

In conclusion, our present study has revealed that PB promoted liver tumors only in *Car*^{+/+} mice, indicating that the nuclear receptor CAR mediates the liver tumor promotion. Once CAR-mediated pathways of tumor promotion are defined at the molecular level, the receptor can be useful as a drug target for HCC prevention.

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