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Xenobiotic Stress Induces Hepatomegaly and Liver Tumors via the Nuclear Receptor Constitutive Androstane Receptor

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The constitutive androstane receptor (CAR, NR1I3) is a central regulator of xenobiotic metabolism. CAR activation induces hepatic expression of detoxification enzymes and transporters and increases liver size. Here we show that CAR-mediated hepatomegaly is a transient, adaptive response to acute xenobiotic stress. In contrast, chronic CAR activation results in hepatocarcinogenesis. In both acute and chronic xenobiotic responses, hepatocyte DNA replication is increased and apoptosis is decreased. These effects are absent in CAR null mice, which are completely resistant to tumorigenic effects of chronic xenobiotic stress. In the acute response, direct up-regulation of Mdm2 expression by CAR contributes to both increased DNA replication and inhibition of p53-mediated apoptosis. These results demonstrate an essential role for CAR in regulating both liver homeostasis and tumorigenesis in response to xenobiotic stresses, and they also identify a specific molecular mechanism linking chronic environmental stress and tumor formation. (*Molecular Endocrinology* 19: 1646–1653, 2005)

WE ARE PROTECTED against potentially harmful effects of foreign compounds, or xenobiotics, by a complex network of drug metabolizing enzymes and regulators (1, 2). The primary regulators are CAR and the pregnane X receptor (NR1I2), which function coordinately to increase expression of a variety of phase I, II, and III metabolic enzymes and transporters in response to xenobiotics (3–6).

CAR is activated by phenobarbital (PB) and a group of structurally diverse agents referred to as "phenobarbital-like" (7). The pesticide contaminant 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) is the most potent PB-like inducer (8) and a specific agonist ligand for murine CAR (mCAR) (9). In contrast, PB and several other inducers do not bind CAR directly, but instead activate a signal transduction pathway that results in translocation of the constitutive transactivator from the hepatocyte cytoplasm to the nucleus (10).

CAR activators can also induce acute hepatomegaly (11, 12). This augments the ability of the liver to clear a xenobiotic stress and could be an adaptive response. However, long-term treatments with these compounds cause liver tumors (11). The xenobiotic inducers differ from other carcinogens in that they do not bind to DNA or cause DNA lesions; instead their effects are thought to be due to their ability to increase cell proliferation and suppress apoptosis (13). In addition to the CAR activators, several other groups of nongenotoxic carcinogens have been identified in rodent assays, but their relevance to human health is controversial due to the lack of a clear molecular mechanism (12–14).

As recently described in independent studies (15), we have found that CAR is essential for tumorigenesis in response to chronic treatment with PB and TCPOBOP. CAR also mediates a transient hepatomegalic response to xenobiotic treatment, which is associated with both induction of DNA replication and suppression of apoptosis. CAR directly activates Mdm2 expression, and loss of Mdm2 function blunts the replicative response to TCPOBOP. The ability of human (h) CAR to induce similar effects in the mouse suggests that this receptor may mediate the effects of chronic xenobiotic stress on hepatocarcinogenesis in humans.

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Abbreviations: CAR, Constitutive androstane receptor; DEN, N-nitrosodiethylamine; h, human; mCAR, murine CAR; PB, phenobarbital; TCPOBOP, contaminant 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; RXR, retinoid X receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

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RESULTS

CAR Mediates Reversible Hepatomegaly in Response to Acute Xenobiotic Stress

Previous results showed that CAR is required for the enlargement of liver size and the induction of DNA synthesis by PB and TCPOBOP (16). To further define this response and its potential relationship to liver

tumor promotion, we examined the effects of acute xenobiotic treatment and withdrawal in wild-type and $CAR^{-/-}$ mice. PB is cleared rapidly (17), but TCPOBOP persists in the liver (18). Expression of the CAR target gene *Cyp2B10* was strongly induced by 3 d of treatment with either xenobiotic, and this induction was absent 3 d after cessation of treatment with PB, but not TCPOBOP (Fig. 1A). Both xenobiotics increased liver size in wild-type, but not $CAR^{-/-}$ mice, as expected, and this response was also significantly decreased 3 d after withdrawal of PB, but not TCPOBOP (Fig. 1B). The recovery of liver size after withdrawal of PB treatment was associated with increased apoptosis in wild-type mice (published as supplemental Fig. 1 on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).

The majority of hepatocytes are tetraploid under ordinary circumstances, with lesser numbers of diploid and octoploid cells. Cell sorting revealed that PB or TCPOBOP treatment increased the ratio of octoploid to tetraploid hepatocytes in wild-type mice. This endoreduplication was not observed in $CAR^{-/-}$ mice, and the 8N/4N ratio decreased significantly in wild-type mice after withdrawing PB, but not TCPOBOP (Fig. 1C).

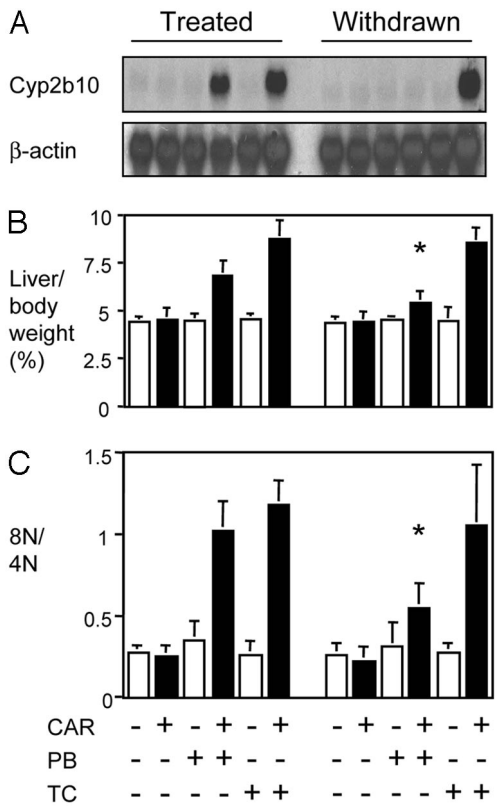

Fig. 1. Role of CAR in Acute Xenobiotic Stress and Withdrawal

A, Cyp2b10 expression in total liver RNA from three to four male wild-type or $CAR^{-/-}$ mice either treated with PB or TCPOBOP (TC) for 3 d or treated and withdrawn for 4 d. B, Relative liver weight of mice either treated with PB or TC or treated and withdrawn. (*, $P < 0.05$ relative to PB treated.) C, The ratio of octoploid to tetraploid primary hepatocytes (8N/4N) in livers from mice either treated with PB or TC or withdrawn, as indicated, was determined by flow cytometry. (*, $P < 0.01$ relative to PB treated.)

We conclude that CAR directs a transient replicative response to an acute xenobiotic stress. The increased liver size should promote clearance of the stress, particularly because an increase in hepatocyte ploidy has been associated with higher metabolic activity (19).

CAR Mediates Hepatocarcinogenesis in Response to Chronic Xenobiotic Stress

Although hepatomegaly can thus be considered an adaptive response to acute stress, both PB and TCPOBOP are nongenotoxic hepatocarcinogens. We compared the responses of wild-type and $CAR^{-/-}$ mice to extended PB or TCPOBOP exposure, with or without prior treatment with the alkylating agent N-nitrosodiethylamine (DEN). In accord with previous results (11), treatment of wild-type mice with a single nontumorigenic dose of DEN followed 2 wk later by 30 wk of subsequent xenobiotic treatment resulted in a large number of tumors (Fig. 2A and Table 1). Both adenomas and carcinomas were observed in DEN plus PB and also DEN plus TCPOBOP-treated animals (Fig. 2B and Table 1). Although tumors can be induced by longer-term PB treatments, none were observed with PB or DEN alone in these studies. TCPOBOP induced liver adenomas, as expected (11). In striking contrast, none of the treatment combinations resulted in any tumors in the $CAR^{-/-}$ mice (Fig. 2A and Table 1). This requirement of CAR for xenobiotic-induced tumorigenesis is very consistent with results of a recent independent study of PB effects that used a different $CAR^{-/-}$ line (15).

To investigate whether chronic xenobiotic stress affects hepatocyte proliferation and apoptosis, we compared both responses in wild-type and $CAR^{-/-}$ animals after the long-term TCPOBOP treatment. As expected and as reported for chronic PB treatment (15), chronic administration of TCPOBOP to the wild-type mice resulted in increased expression of Cyp2B10 and other CAR targets (supplemental Fig. 2A). Chronic xenobiotic stress was also associated with increased DNA replication, as shown by specific increases in the hepatoctye 8N/4N ratio (Fig. 2C) and in the number of PCNA-positive cells in the wild-type TCPOBOP-treated mice (supplemental Fig. 2B). Consistent with reported acute anti-apoptotic effects of PB (20), liver sections from the chronically TCPOBOP-treated wild-type mice showed significantly lower numbers of apoptotic cells than the untreated controls or $CAR^{-/-}$ mice (Fig. 2D). Thus, chronic and acute xenobiotic stresses produce similar responses.

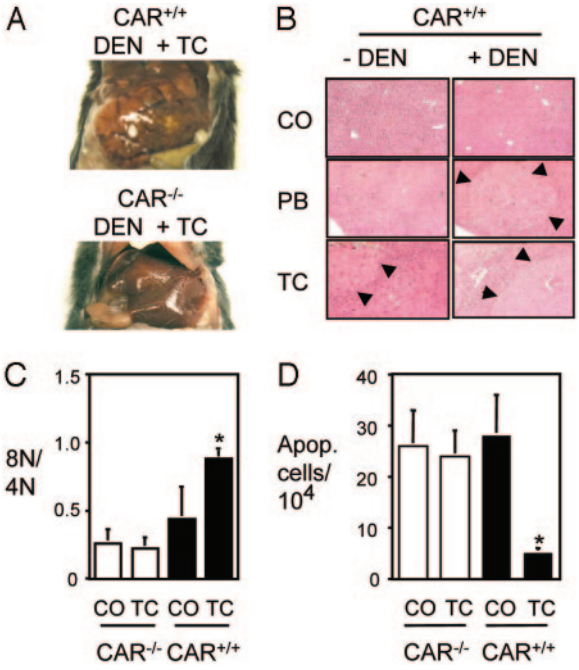

Fig. 2. CAR Is Required for Induction of Tumors by Chronic Xenobiotic Stress

A, Representative liver morphology of wild-type or *CAR*$^{-/-}$ mice treated with a single injection of DEN followed by TCPOBOB treatment for 30 wk. B, Histological analysis of liver sections from individual wild-type mice with indicated treatments. *Arrows* indicate tumor areas. C, Ratio of 8N to 4N cells in livers from wild-type and *CAR*$^{-/-}$ mice with or without long-term TCPOBOP treatment. (*, $P < 0.01$ relative to untreated wild type.) D, The numbers of cells undergoing apoptosis in livers of wild-type and *CAR*$^{-/-}$ mice with or without long-term TCPOBOP treatment was determined by TUNEL assay. (*, $P < 0.05$ relative to untreated wild type.)

CAR Activation Produces a Tumorigenic Environment via Induction of Mdm2

The replicative increase in ploidy upon acute CAR activation is associated with increased expression of a number of cell cycle regulators (Ref. 21 and data not shown), but none have been identified as primary CAR targets. Gene array screens for potential CAR targets rapidly induced by TCPOBOP identified Mdm2 among a number of other targets. Mdm2 is of particular interest because it suppresses p53-dependent apoptosis and can also stimulate cell proliferation in a p53-independent manner (22). Moreover, recent studies have linked increased Mdm2 expression to the formation of preneoplastic lesions in the liver (23), and overexpression of Mdm2 has been observed in human hepatocellular carcinoma (24). Northern blotting confirmed induction of Mdm2 by TCPOBOP (Fig. 3A), and both quantitative real-time PCR (data not shown) and Western blot analysis (Fig. 3B) showed that TCPOBOP treatment increased hepatic Mdm2 expression at early time points in wild-type but not CAR$^{-/-}$ mice.

A previous report identified an element in the first intron of the *Mdm2* gene that confers response to thyroid hormone (25). CAR/retinoid X receptor (RXR) heterodimers also bind this DR4 element, but not a mutant version, with an affinity comparable to but somewhat less than that of the previously characterized DR5 site from the RARβ promoter (Fig. 3C). In transient transfections, CAR transactivated a reporter construct containing the functional *Mdm2* element, but not the mutant (Fig. 3D). Chromatin immunoprecipitation demonstrated specific binding of CAR to this site in the endogenous *Mdm2* gene in a stable CAR expressing $HepG_2$ derivative, but not parental $HepG_2$ cells (Fig. 3E). As in other cell lines, CAR is constitutively nuclear in these cells. We conclude that *Mdm2* is a primary CAR target gene.

Loss of Mdm2 function results in embryonic lethality that can be suppressed by loss of p53 (26, 27). Because the loss of p53 function does not affect the proliferative response to PB (28), we investigated the potential role of Mdm2 in TCPOBOP-induced hepatomegaly by treating *p53*$^{-/-}$ and *p53*$^{-/-}$/*Mdm2*$^{-/-}$ double-null mice with a single injection of TCPOBOP. Loss of Mdm2 function in this context blunted the TCPOBOP-induced replicative response as demon-

Table 1. Effects of PB and TCPOBOP on Hepatocarcinogenesis in Wild-Type and CAR Null Mice

	CAR$^{+/+}$			CAR$^{-/-}$		
	Liver/Body Weight (Mean ± SD)	Percentage of Mice with Tumor[a]	Tumors/Tumor Bearing Mouse (Mean ± SD)	Liver/Body Weight (Mean ± SD)	Percentage of Mice with Tumor[a]	Tumors/Tumor Bearing Mouse (Mean ± SD)
Vehicle	4.37 ± 0.39	0	0	4.11 ± 0.4	0	0
Vehicle/PB	5.51 ± 0.45[b]	0	0	4.14 ± 0.6	0	0
Vehicle/TC	6.99 ± 0.95[b]	44	3.3 ± 1.7	4.16 ± 0.9	0	0
DEN	4.04 ± 0.57	0	0	3.76 ± 0.4	0	0
DEN/PB	6.00 ± 0.81[b]	78[c]	6.4 ± 3.2[d]	4.03 ± 0.7	0	0
DEN/TC	18.48 ± 6.67[b]	100[c]	>12[d]	3.85 ± 0.40	0	0

TC, TCPOBOP.
[a] Includes only animals surviving until termination of experiment.
[b] $P < 0.05$ compared with vehicle or DEN wild-type animal group.
[c] $P < 0.05$ compared with vehicle/TC wild-type animal group.
[d] $P < 0.05$ compared with vehicle/TC wild-type animal group.

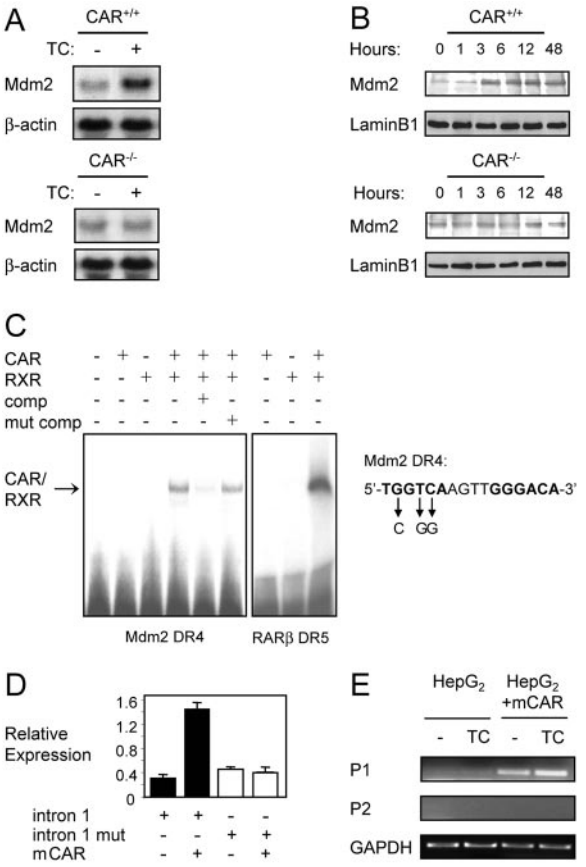

Fig. 3. Mdm2 as a CAR Target Gene

A, Total liver RNA pooled from three control (−) or TCPOBOP (TC)-treated wild-type or *CAR*$^{-/-}$ mice was used for Northern analysis with the indicated probes. B, Nuclear extracts were prepared from TCPOBOP-treated wild-type or *CAR*$^{-/-}$ livers at indicated time points and analyzed by Western blot with a Mdm2 monoclonal antibody or an anti-laminB1 antibody. C, Binding of CAR and RXR to the indicated DR-4 containing oligonucleotide from the first intron of the *Mdm2* gene was assessed by electrophoretic mobility shift. Unlabeled DR-4 oligonucleotide or the indicated mutant version were used as competitors as indicated. D, Hela cells were transfected with a TK-CAT reporter plasmids containing a single copy of the wild-type or mutant Mdm2 DR-4 element, with or without CAR expression vector. E, Parental $HepG_2$ and $HepG_2$-mCAR cell lines were treated with solvent (−) or 500 nM TCPOBOP (TC) for 3 h. Chromatin immunoprecipitation was carried out using an anti-Flag tag antibody that recognizes the tagged mCAR and PCR primers flanking the DR-4 sequence (P1) or a segment 10 kb upstream (P2). Five percent of DNA input was subjected for a PCR with a pair of glyceraldehyde-3-phosphate dehydrogenase-specific primers. As in other cell lines, CAR is constitutively nuclear in $HepG_2$ cells.

strated by decreases in the proportion of octoploid hepatocytes (Fig. 4) and the number of PCNA-positive nuclei (supplemental Fig. 3). These results indicate that Mdm2 contributes to TCPOBOP-induced endoreduplication but also strongly suggest that other genes are involved.

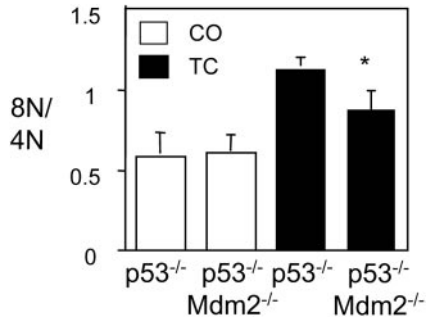

Fig. 4. Role of Mdm2 in CAR-Dependent Endoreduplication

Primary hepatocytes were isolated from *p53*$^{-/-}$ or *p53*$^{-/-}$ *Mdm2*$^{-/-}$ mice treated with corn oil (CO) or one dose of TCPOBOP (TC) for 3 d. The ratio of octoploid and tetraploid cells (8N/4N) was determined by flow cytometry. (*, $P < 0.05$.)

Mdm2 induction should inhibit p53-dependent apoptotic pathways, and TCPOBOP treatment of wild-type primary hepatocytes strongly suppressed UV-induced apoptosis (Fig. 5A), in agreement with recent results with other PB-like activators (29). This suppression was completely absent in *CAR*$^{-/-}$ cells. TCPOBOP also suppressed apoptosis and induced Mdm2 mRNA in a CAR expressing $HepG_2$ cell line, but not parental $HepG_2$ cells (supplemental Fig. 4, A and B). CAR expression in the HepG2 derivative resulted in a strong decrease in both basal and UV-induced p53 protein levels (supplemental Fig. 4C).

CAR activation can also suppress apoptosis induced by other DNA damaging agents. Consistent with previous results with PB (30), TCPOBOP strongly decreased bleomycin-induced apoptosis in wild-type, but not *CAR*$^{-/-}$ primary hepatocytes (Fig. 5B). The response to this chemotherapeutic agent, which induces double-strand breaks, is clearly dependent on p53 because it did not induce apoptosis in *p53*$^{-/-}$ primary hepatocytes. TCPOBOP also blocked apoptosis induced by a single ip injection of the alkylating agent methyl methanesulfonate in the livers of wild-type but not *CAR*$^{-/-}$ mice (Fig. 5C). As with the bleomycin-treated primary hepatocytes, the basal response observed in the TCPOBOP-treated wild-type mice was indistinguishable from that observed in *p53*$^{-/-}$ mice. Thus, both cell culture and *in vivo* experiments demonstrate a selective suppression of p53-mediated apoptosis upon CAR activation.

Replicative and Antiapoptotic Effects of hCAR

To determine whether hCAR can induce similar replicative and antiapoptotic responses, we used a previously described mouse strain that expresses only hCAR in the liver (31). hCAR is not responsive to TCPOBOP, but treatment with PB for 1 wk induced expression of Cyp2B10 and Mdm2 (Fig. 6A) and significantly increased liver size (Fig. 6B). Both the proportion of octoploid hepatocytes and the number of PCNA-positive cells were also increased (Fig. 5C). PB

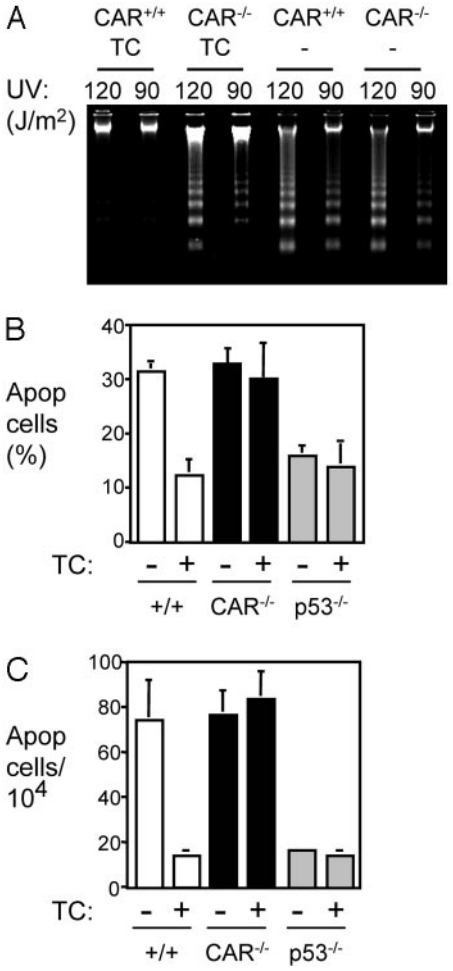

Fig. 5. CAR Activation Suppresses p53-Mediated Apoptosis

A, Primary hepatocytes from either wild-type or $CAR^{-/-}$ mice were UV irradiated (90 J/m^2 and 120 J/m^2) followed by the treatment with solvent (CO) or TCPOBOP (TC, 1 μM) for 24 h. DNA fragmentation was used to test for apoptosis. B, Primary hepatocytes isolated from wild-type (+/+), $CAR^{-/-}$ or $p53^{-/-}$ mice were pretreated with solvent (−) or 500 nM TCPOBOP (TC) for 12 h. Cells were treated with 15 μg/ml bleomycin for 24 h. The percentage of apoptotic cells was determined by TUNEL assay. Results are from three independent experiments. C, Wild-type (+/+), $CAR^{-/-}$ or $p53^{-/-}$ mice were injected with a single dose of corn oil (CO) or TCPOBOP (TC, 3 mg/kg). After 3 d, methyl methanesulfonate (1.5 mmol/kg) was injected ip and livers were removed after 3 h and fixed. Apoptotic cells were identified by TUNEL assay and counted in three different samples for each genotype.

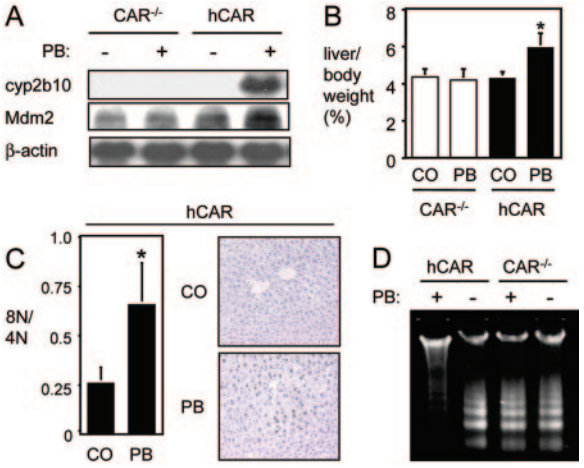

Fig. 6. Responses of hCAR Mice

A, Gene expression in PB or control fed (0.05% PB diet for 1 wk) hCAR and $CAR^{-/-}$ mice. B, Liver size of PB or control fed hCAR and $CAR^{-/-}$ mice. (*, $P < 0.05$.) C, Proportion of octoploid hepatocytes (*, $P < 0.01$) and PCNA-positive cells in PB or control-fed hCAR mice. D, UV-induced apoptosis in primary hepatocytes isolated from hCAR and $CAR^{-/-}$ mice.

treatment of primary hepatocytes from the hCAR mice also suppressed UV-induced apoptosis (Fig. 5D). Thus, activation of hCAR in the mouse background generates all of the acute responses observed with mCAR.

DISCUSSION

The current results confirm and extend previous studies demonstrating that CAR is required for acute xenobiotic-induced hepatomegaly (16) and are also quite consistent with the recent independent demonstration that it is also essential for hepatocarcinogenesis in response to chronic xenobiotic treatment (15). How are these processes linked at the molecular level? The results described here suggest that CAR directly induces the expression of Mdm2, and likely other central regulators, to activate cell cycle progression and also block apoptosis. This produces a transient hepatomegalic response that promotes xenobiotic clearance in the short term, but clearly also creates a tumorigenic environment.

Multiple steps must be necessary in the progression from the CAR-dependent proliferative state to hepatocellular carcinoma. Such tumor progression has been extensively studied in the liver (32), and foci of proliferating hepatocytes with increased expression of the placental isoform of glutathione-*S*-transferase and other markers are thought to represent a very early stage. It is interesting that glutathione-*S*-transferase-Pi is a CAR target gene (31), that such preneoplastic foci induced by DEN alone or DEN plus PB overexpress Mdm2 (23), and also that withdrawal of PB from chronically treated mice rapidly increases apoptosis in the lesions and decreases their number and size (17, 33). These findings suggest that the proliferative and antiapoptotic environment induced by CAR activators and other nongenotoxic carcinogens may be an important common contributor to early stages of hepatocarcinogenesis. For example, such epigenetic effects could promote the accumulation of cells carrying tumorigenic genetic changes such as the β-catenin mutations that are common in human hepatocarcinomas (34) and also observed in

the majority of mouse liver tumors induced in the presence of PB (35).

The well-known differences in rodent and human xenobiotic responses raise the issue of the relevance of these rodent results to liver carcinogenesis in humans. Preliminary results indicate that chronic xenobiotic stress promotes tumorigenesis in the hCAR mice. Although this is consistent with a limited number of reports linking long-term barbituate treatment to hepatocarcinogenesis (36, 37), long-term barbituate treatment is not associated with increased incidence of liver tumors in humans (38), and similar conclusions have been reached with fibrates and other nongenotoxic agents. This may simply be due to lower doses in humans than in mice. However, humans are relatively resistant to tumorigenesis for a variety of reasons, including shorter telomeres, and the resistance of telomerase-deficient mice to chemically induced hepatocarcinoma (39) is at least consistent with the possibility that such additional mechanisms contribute to the apparent ineffectiveness of the nongenotoxic agents in humans. Overall, as many as 80% of human cancers are thought to be sporadically generated in response to environmental factors (40). Thus, the demonstration of a key role for CAR in tumor promotion provides a novel and potentially important link between environmental stress and tumorigenesis.

MATERIALS AND METHODS

Animals

Mice were hosted in a pathogen-free animal facility under standard 12-h light/12-h dark cycle and fed standard rodent chow and water *ad libitum*. Three to five mice between 8 and 10 wk of age were used in each group of experiments. Treatments with corn oil, PB, and TCPOBOP were as described (16). For the long-term PB and TCPOBOP treatment, at least 10 mice between 4 and 5 wk of age were injected ip with a single dose of DEN (90 mg/kg) followed by feeding with 0.05% PB in powder diet or ip injection of TCPOBOP (3 mg/kg) every 2 wk for 24 wk. All animal experimentation was conducted in accordance with accepted standards of humane animal care.

Cells and Transfections

Hela cells were transfected using calcium phosphate as described (41). Transfections included 100 ng of reporter plasmid, 100 ng β-gal internal control plasmid, and 100 ng CAR expression plasmid. Cells were assayed for chloramphenicol acetyl transferase (Roche Molecular Biochemicals, Indianapolis, IN) activity 24 h after the addition of the ligands, and reporter expression was normalized to the β-gal activity, according to manufacturer' directions. Single nucleotide mutagenesis was performed with QuikChange XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Similar results were obtained from at least three independent experiments. The permanent cell line ($HepG_2$-CAR) was as described (9).

Histology

The left lobe of the livers was removed and fixed in 4% formaldehyde-PBS solution, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Sections were also prepared and stained using a PCNA staining kit (Zymed Laboratories Inc., South San Francisco, CA) or terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) kit (Roche) according to the manufacturers' instruction.

DNA Binding

PT7-lac-His vectors expressing full-length cDNAs of hCAR and hRXRα were used to generate [^{35}S]methionine-labeled proteins by *in vitro* translation as described (9). End-labeled double-stranded oligonucleotides were incubated with 1–2 μl of [^{35}S]methionine-labeled CAR and RXR. Complexes were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel and visualized by autoradiography.

DNA Fragmentation

Mouse primary hepatocytes were prepared and cultured as described (42). Primary hepatocytes were plated at a density of 60,000 cells/cm^2 and treated with UV at indicated doses after 12 h. Cells were collected 24 h after UV treatment and washed with PBS. The cell sediment was resuspended in 2.5 ml cell lysis buffer (50 mM Tris, 1 mM EDTA, 1% sodium dodecyl sulfate) and 10 μl 1 mg/ml proteinase K at 37 C for 1 h and DNA was prepared by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Ten micrograms of DNA per sample were resolved on a 1.8% agarose gel.

Protein Analysis

Freshly excised liver specimens were homogenized in buffer [2 M sucrose, 10 mM HEPES (pH 7.6), 25 mM KCl, 1 mM EDTA, 10% glycerol, 0.15 mM spermine, 1 mM spermidine, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstain A, 0.1 mM Pefabloc, and 50 μg/ml N-acetylleucylleucylnorleucinal] and were centrifuged at 24,000 rpm for 1 h at 4 C. The nuclear pellet was resuspended in 0.5–0.7 ml extraction buffer [10 mM HEPES (pH 7.6), 100 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstain A, 0.1 mM Pefabloc, 50 μg/ml N-acetylleucylleucylnorleucina]. A 1/10 volume 4M $(NH4)_2SO_4$ (pH 7.9) was added, and the mixture was gently agitated at 4 C for 1 h. The lysate was centrifuged at 85,000 rpm for 1 h, and the clean supernatant was the nuclear extract. Liver nuclear extracts (40 μg) were resolved by 10% PAGE and immunoblotted with antibodies specific for Mdm2 (Oncogene) and laminB1 (Zymed Laboratories Inc.). For p53, 300 μg total protein from cell lysates were immunoprecipitated and blotted with a p53 monoclonal antibody (Oncogene, Cambridge, MA). Western blotting was performed using ECL kit (Amersham Biosciences, Piscataway, NJ).

RNA Analysis

Total liver RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instruction. Equivalent amounts of RNA from three to five mice were pooled and 10–15 μg was subjected to Northern blot analysis. All cDNA probes were prepared by RT-PCR with mouse liver RNA using Super-Script One Step RT-PCR system (Invitrogen). PCR primers used were: human Mdm2, atggtgaggagcaggtactg and ccaatcgccactgaacacag.

Flow Cytometry

Primary hepatocytes were prepared and 2×10^6 cells were resuspended in 2 ml 0.9% NaCl. Five milliliters of 90% cold

EtOH was added drop wise to fix the cells for at least 30 min at room temperature. Before they were subjected to flow cytometry, cells were incubated with 100 μl 1 mg/ml ribonuclease and stained with 50 μg/ml propidium iodide at 37 C for 30 min. Cell sorting was performed at the core facility at Baylor College of Medicine.

Chromatin Immunoprecipitation

$HepG_2$ or $HepG_2$-mCAR cells were treated with either solvent or 500 nM TCPOBOP for 3 h. Cells were collected and chromatin complexes were prepared and immunocleared with protein A/G agarose and 2 μg/ml sheared salmon sperm DNA for 2 h at 4 C. The cleared chromatin complexes were immunoprecipitated overnight at 4 C with an anti-Flag antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) against the mCAR epitope tag, followed by protein A/G agarose at room temperature for 2 h. PCR analysis was performed using a pair of Mdm2 intron1-specific primers (P1): forward primer, ttcagtgggcaggttgac and reverse primer, acaagtcaggacttaactcc. A pair of primers amplifying a fragment approximately 10 kb upstream of Mdm2 intron1 (P2) were used as a negative control: forward, ttcatgcaattctcctgc and reverse, tcaggagttcgagaccag. Five percent of input chromatin complexes were subjected to a loading control PCR using a pair of glyceraldehyde-3-phosphate dehydrogenase-specific primers.

Statistics

The values were represented as mean ± SEM. Statistical analysis was carried out using two-tailed Student's *t* test.

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