RETINOIC ACIDS REPRESS CONSTITUTIVE ACTIVE RECEPTOR-MEDIATED INDUCTION BY 1,4-BIS[2-(3,5-DICHLOROPYRIDYLOXY)]BENZENE OF THE CYP2B10 GENE IN MOUSE PRIMARY HEPATOCYTES

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ABSTRACT:

The nuclear orphan receptor constitutive active receptor (CAR) can be activated to induce CYP2B genes by the potently phenobarbital-type inducer 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) in which the receptor forms a heterodimer with the retinoid X receptor (RXR) and binds to a conserved enhancer element NR1. Effects of retinoic acids on the activation of CAR were examined. Treatment with 9-cis- or all-trans-retinoic acid markedly repressed TCPOBOP induction of CYP2B10 mRNA in mouse primary hepatocytes. Both retinoic acids also repressed TCPOBOP-induced NR1 enhancer activity in both transfected hepatocytes and HepG2 cells. Moreover, coexpression of the retinoic acid receptor (RAR) increased the repression in the cotransfected HepG2 cells, whereas that of RXR decreased the repression. Thus, the increased heterodimerization of RXR with RAR by retinoic acid treatment seemed to reduce the RXR available for CAR heterodimerization, resulting in the repression of CAR activity. This type of nuclear receptor signaling may play an important role as a modulator in the CYP2B regulation.

Cytochromes P450 (P450s1) play a major role in the detoxification and activation of therapeutic drugs and xenocarcinogens. Inducible gene transcription by exposure to xenocarcinogens is characteristic for P450s and increases the metabolic capabilities against drug and chemical toxicity (Nebert and Gonzalez, 1987; Waxman and Azaroff, 1992). Phenobarbital (PB) is the prototype for a large number of structurally diverse chemicals that induce P450s and other xenocarcinogen-metabolizing enzymes (Waxman and Azaroff, 1992; Honkakoski et al., 1998a). Nuclear orphan receptors have recently emerged as important P450 gene regulators and their cross talk becomes an important focus for understanding the induction mechanisms.

The nuclear orphan receptor CAR was originally characterized as a receptor that activates an empirical set of retinoic acid response elements without the presence of retinoic acids (Baes et al., 1994; Choi et al., 1997). The first gene identified as a direct target of CAR is the CYP2B genes of mouse, rat, and human, which are induced in response to PB treatment (Honkakoski et al., 1998a; Sueyoshi et al., 1999). Treatment with PB translocates CAR from the cytoplasm into the nucleus of mouse livers and primary hepatocytes (Kawamoto et al., 1999). Forming a heterodimer with RXR in the nucleus, CAR binds to the NR1 site within the conserved 51-bp PBREM (phenobarbital-responsive enhancer module) found in the mouse and human CYP2B genes (Honkakoski et al., 1998a,b; Kawamoto et al., 1999; Sueyoshi et al., 1999). CAR dimerizes with RXR in the absence of retinoic acids (Baes et al., 1994; Choi et al., 1997). A role for retinoic acids in the CAR/RXR heterodimer remains an intriguing question that may be critical for modulating P450 induction. Yamada et al. (2000) have recently reported that 9-cis- and all-trans-retinoic acids markedly repressed CYP2B1/2 induction by PB in rat primary hepatocytes. In the present studies, the effects of retinoic acids on CAR-mediated induction of the CYP2B gene has been further examined using CAR-transfected HepG2 cells and/or mouse primary hepatocytes. The experimental considerations will be presented to suggest that retinoic acids decrease availability of RXR for CAR by heterodimerization, promoting interactions with other receptors, such as the retinoic acid receptor (RAR).

Experimental Procedures

Materials. The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): 9-cis-retinoic acid and all-trans-retinoic acid. Stock solutions of retinoic acids were prepared in dimethyl sulfoxide (DMSO). 1,4-bis[2-(3,5-Dichloropyridyloxy)]benzene (TCPOBOP) was synthesized using the method of Kende et al. (1985). The expression plasmids RSV-RARs and pCMV-RXRα were kindly provided by Ronald Evans (The Salk Institute for Biological Studies, La Jolla, CA). (NR1)-tk (thymidine kinase)-luciferase plasmid was constructed by cloning quintuple NR1 sequences in front of the tk-luciferase promoter (at the Bgl II site), as described previously (Sueyoshi et
plasmids (0.1 μg/H9262). Rabbit IgG-horseradish peroxidase conjugate, the immunoreactive bands were incubated with anti-CAR antibody. After incubation with the secondary anti-Dignam et al. (1983). Nuclear extracts were resolved on a SDS-10% poly-
prepared from these hepatocytes after a 1-h treatment by the method of
Because the CAR/RXR heterodimer is essential for the
been associated with the function of CAR, the repression mechanism
the constitutive expression of the CYP2B10 mRNA. Thus, the similar repression by
CYP2B1/2 in PB-treated rat primary hepatocytes, consistent with our
findings for the CYP2B10 mRNA. The level of CYP2B10
mRNA were normalized by the GAPDH mRNA levels and are expressed as a -fold
induction taking the control value as 1. B, mouse primary hepatocytes were treated
with the indicated retinoic acids (10 μM) and TCPOBOP (50 nM) for 8 h. The level of
CYP2B10 mRNA are expressed as a -fold induction taking the noninduced
control value as 1.

Transfection Assays. HepG2 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum. Mouse CAR (mCAR)
expression plasmids (0.1 μg) were cotransfected with (NR1)_5-tk-luciferase plasmids (0.1 μg) and pRL-SV40 (0.1 μg) into HepG2 cells (17-mm well) by the calcium phosphate coprecipitation method using a CellPhect transfection kit (Amersham Biosciences, Piscataway, NJ). RAR and RXR plasmids were also cotransfected with indicated concentrations. Mouse primary hepatocytes were prepared and (NR1)_5-tk-luciferase plasmids (10 μg) were cotransfected with pRL-SV40 (5 μg) into hepatocytes using electroporation. These cells were treated with chemicals or the solvent (DMSO) for 24 h at the indicated concentrations, and luciferase activity was measured by using the dual-luciferase reporter assay system (Promega, Madison, WI).

Western Blot. Mouse primary hepatocytes were treated with retinoic acids (10 μM), TCPOBOP (50 nM), or the solvent (DMSO). Nuclear extracts were prepared from these hepatocytes after a 1-h treatment by the method of Dignam et al. (1983). Nuclear extracts were resolved on a SDS-10% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and incubated with anti-CAR antibody. After incubation with the secondary anti-rabbit IgG-horseradish peroxidase conjugate, the immunoreactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences).

Results and Discussion

Inhibition of CYP2B10 mRNA in Primary Hepatocytes. Both 9-cis- and all-trans-retinoic acids (10 μM) slightly repressed CYP2B10 mRNA in mouse primary hepatocytes (Fig. 1A). The repression was profound in the TCPOBOP-induced (50 nM) hepatocytes in which both retinoic acids repressed the mRNA to less than 30% of the induced levels (Fig. 1B). Yamada et al. (2000) reported Western blot analysis showing the repression by retinoic acids of CYP2B1/2 in PB-treated rat primary hepatocytes, consistent with our findings for the CYP2B10 mRNA. Thus, the similar repression by retinoic acids was observed in both rat and mouse hepatocytes. Since the constitutive expression of the Cyp2b10 gene in mouse liver has not been associated with the function of CAR, the repression mechanism by retinoic acid is difficult to speculate about at the present time. Because the CAR/RXR heterodimer is essential for the Cyp2b10 gene being induced by TCPOBOP or PB, we performed an in vitro transfection assay to demonstrate that the repression can, in fact, be regulated through RXR.

Inhibition of NR1 Activity. To investigate whether the retinoic acid-induced repression of CYP2B10 mRNA is correlated with that of NR1 activity, a (NR1)_5-tk-luciferase plasmid was transfected into mouse primary hepatocytes. After being induced with TCPOBOP (50 nM), the hepatocytes were treated with 9-cis- or all-trans-retinoic acids (10 μM) and subjected to luciferase assay (Fig. 2A). Consistent with the observation for the CYP2B10 mRNA, both retinoic acids repressed the induced NR1 activity. It is known that an initial step of CAR activation by PB is its nuclear translocation in mouse primary hepatocytes (Kawamoto et al., 1999). Treatment with retinoic acids, however, did not inhibit the nuclear accumulation in the TCPOBOP-induced hepatocytes, as shown in Fig. 2B. Western blot analysis,
Acids (10 nM). These results suggested that the repression of CAR occurs in the nucleus.

A, a (NR1)tk-luciferase plasmid was cotransfected with pRL-SV40 into mouse primary hepatocytes. After being induced with TCP0BOP (50 nM), the hepatocytes were treated with 9cis- or all-trans-retinoic acids (10 nM) and subjected to luciferase assay. The NR1 enhancer activity level is indicated as a fold induction taking the noninduced control value as 1. B, mouse primary hepatocytes were treated with the indicated retinoic acids (10 nM) and TCP0BOP (50 nM) for 1 h. Nuclear extracts were prepared from these cells and subjected to Western blot analysis using anti-CAR antibody. Prestained protein marker broad range (New England Biolabs, Inc., Beverly, MA) was used as the molecular marker.

Using anti-CAR antibody, was performed on the nuclear extracts prepared from the differently treated hepatocytes. Treatment with 9cis- or all-trans retinoic acid (10 nM) neither caused the nuclear accumulation of CAR nor inhibited that induced by TCP0BOP (50 nM). These results suggested that the repression of CAR occurs in the nucleus.

NR1 Repression by Retinoic Acids in HepG2 Cells. HepG2 cells and transient transfection assays were used for further investigation of the repression mechanism of CAR by retinoic acids, taking advantage of the fact that the receptor localizes in the nucleus and activates NR1 in the cotransfected cells (Kawamoto et al., 1999). Although retinoic acids (10 nM) repressed the CAR-mediated NR1 activity in the nontreated cells (data not shown), we performed all experiments using the TCP0BOP (250 nM) pretreatment cells in which a higher NR1 activity enhanced the degree of repression. First, we examined the effects of an increased level of the exogenous RAR on the NR1 activity in CAR-transfected HepG2 cells. The NR1 activity was decreased as the RAR levels increased and was completely repressed at its highest level observed experimentally (Fig. 3A). Consistent with RAR being a limiting factor for regulating the NR1 activity, the additional coexpression of RAR together with RAR restored some of the repressed NR1 activity in the TCP0BOP-treated cells, whereas the RAR alone did not affect the activity (Fig. 3B). Moreover, the retinoic acid-repressed NR1 activity was also restored by the exogenous RAR in the cotransfected HepG2 cells (Fig. 3C). Although 9cis-retinoic acid seemed to be slightly more effective in repressing the NR1 activity (data not shown), there was no fundamental difference in the repressing ability compared with all-trans retinoic acid. These results are consistent with the hypothesis that the retinoic acids activate RAR and increase the heterodimerization with RXR, thereby exhausting the RAR available for CAR and resulting in the repression of the NR1 activity.

The pattern of gene expression in RAR-null mice has shown that this nuclear receptor plays the central role in many biological processes as the common heterodimerization partner for various nuclear receptors (Wan et al., 2000). CAR is one of many nuclear receptors that require RAR as a dimerization partner, thus competing for a pool of RAR with other nuclear receptors. CAR is heterodimerized with RAR in the absence of PB or retinoic acids. However, only in response to PB exposure does CAR activate PBREM and induce the Cyp2b10 gene in mice livers and mouse primary hepatocytes (Honkakoski et al., 1998a, b; Kawamoto et al., 1999; Sueyoshi et al., 1999). Our present studies have shown that retinoic acids repress the CAR-mediated PBREM activity and the TCP0BOP induction of the Cyp2b10 gene in mouse primary hepatocytes. Retinoic acids at a pharmacological level in the presence of RAR could indirectly repress the CAR activity by reducing the availability of RAR for CAR (Fig. 4). This type of receptor cross talk (i.e., competition among various receptors for RXR).
receptors for heterodimerization with RXR) may be a critical role in drug efficacy and drug-drug interactions.

References


