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 potent 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 (P450s) play a major role in the detoxification and activation of therapeutic drugs and xenoc hemicals. Inducible gene transcription by exposure to xenoc hemicals 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 xenoc hemical-metabolizing enzymes (Waxman and Azaroff, 1992; Honkakoski et al., 1998a). Nuclear orphan receptors have recently emerged as important 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 gene 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 BglII site), as described previously (Sueyoshi et
al., 1999). Production of anti-CAR antiserum and other recombinant plasmids was previously described (Kawamoto et al., 1999; Suyeoshi et al., 1999).

RT-PCR. Mouse primary hepatocytes were prepared from 2-month-old Crl:CD-1(ICR)BR males by a two-step collagenase perfusion and were cultured as previously described (Honkakoski et al., 1998b). Total RNAs were extracted from the hepatocytes for RT-PCR analysis 8 h after treatment with chemicals or the solvent (DMSO) at the indicated concentration, using TRIZOL reagent (Invitrogen, Rockville, MD). To quantify CYP2B10 mRNA, cDNA was prepared from total cellular RNA of mouse primary hepatocytes using the SuperScript preamplification system for first-strand cDNA synthesis (Invitrogen). cDNA was subjected to quantitative real-time PCR using ABI Prism 7700 (Applied Biosystems, Foster City, CA). CYP2B10 cDNA was amplified using 5'-AAAGTCCCGTGGCAACTTCC-3' and 5'-TCCCCGCTGGCAACTTCC-3' for 5' - and 3'-primers, respectively. Amplified cDNA was measured using 6FAM-ACCCCGTCCCCCTGCCCTT-TAMRA as a CYP2B10 probe. For an internal control, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level was also measured using TaqMan rodent GAPDH control reagents (Applied Biosystems). The quantity of the CYP2B10 mRNA level was normalized by simultaneously measuring the GAPDH mRNA level and was indicated as a -fold induction of control.

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 (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 10% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and 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.

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.
A, a (NR1),-tk-luciferase plasmid was cotransfected with pRL-SV40 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. 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 μM) and/or TCPOBOP (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 9-cis- or all-trans retinoic acid (10 μM) neither caused the nuclear accumulation of CAR nor inhibited that induced by TCPOBOP (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 μM) repressed the CAR-mediated NR1 activity in the nontreated cells (data not shown), we performed all experiments using the TCPOBOP (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 RXR together with RAR restored some of the repressed NR1 activity in the TCPOBOP-treated cells, whereas the RXR alone did not affect the activity (Fig. 3B). Moreover, the retinoic acid-repressed NR1 activity was also restored by the exogenous RXR in the cotransfected HepG2 cells (Fig. 3C). Although 9-cis-retinoic acid seemed to be slightly more effective in repressing the NR 1 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 RXR available for CAR and resulting in the repression of the NR1 activity.

The pattern of gene expression in RXR-null mice has shown that this nuclear receptor plays the central role in many biological pro-
receptors for heterodimerization with RXR) may be a critical role in drug efficacy and drug-drug interactions.

References


