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Suppression of the Expression of the CYP2B1/2 Gene by Retinoic Acids

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The effects of 5α-androsten-3α-ol (ASE), and retinoic acids (RAs) and their precursors on the phenobarbital (PB)-mediated induction of CYP2B1 and 2B2 were examined in cultured rat hepatocytes. Two isomers of RA, 9-*cis*- and all-*trans*-RA, suppressed markedly the effect of PB on CYP2B1/2 expression, while ASE had no suppressive effect. The effect of 9-*cis*-RA appeared at a lower concentration than the all-*trans*-isomer, indicating the dominant action of the former isomer. Suppression with 9-*cis*-retinal was also observed, but all-*trans*-retinol and -retinal were without effect. These results suggest that: (1) ASE, an inverse agonist for the constitutive androstane receptor (CAR), does not play a major role in the suppression of the CYP2B; (2) 9-*cis*-RA suppresses CYP2B induction by reducing ligand-free retinoid X-receptors (RXR) available for dimerization with the CAR; and (3) enzymes responsible for RA formation play an important role in the mechanism governing CYP2B regulation.

Key Words: **CYP2B1; CYP2B2; phenobarbital; retinoic acid; constitutive androstane receptor; retinoid X-receptor.**

Hepatic P450 cytochromes (P450) play a major role in the detoxication and activation of xenobiotics. Of these P450s, the CYP2B subfamily have been studied extensively due to their highly inducible nature (1) and wide range of substrate specificity (2). The mechanism by which phenobarbital (PB) induces the CYP2B subfamily P450 is not yet fully understood (3–5). Recent results suggest that the constitutive androstane receptor (CAR) and retinoid X-receptor (RXR) play a crucial role in the regulation of the CYP2B genes (6–8). 5α-Androsten-3α-ol (ASE), one of the endogenous CAR ligands, has been shown to act as an inverse agonist, suppressing the transactivating effect of the CAR by binding to this receptor (7–9). It is, therefore, likely that the expression of the CYP2B enzymes in liver is suppressed by ASE in the absence of exogenous inducer and this suppressive control is removed by the inducer. Quite recently, TCPOBOP, an inducer of CYP2B, but not PB has been shown to bind to the CAR with a much higher affinity than ASE at the same site for ASE (10). This observation raises the question: Why do ASE and TCPOBOP exhibit opposite effects although they occupy the same receptor site? In addition, if the role of PB in induction is recruitment of the CAR as proposed (8), ASE should suppress PB-mediated induction by binding to the CAR accumulated in the nucleus. To our knowledge, this point remains to be examined. Thus, the regulatory mechanism and the effect of ASE on CYP2B induction need further clarification.

It has been demonstrated that neither CAR-RXR dimerization nor transactivation of the RA-responsive element (RARE) by this complex requires the presence of RA (11, 12). The RARE consists of two repeated nucleotide sequences separated by 5 bases (direct repeat-5; DR-5) (11). However, the requirement of RA remains obscure in the CAR/RXR heterodimer-dependent transactivation of PBRU or PBREM, a DR-4 type DNA element which plays a role in PB-mediated induction of the CYP2B subfamily (13, 14). Indeed, even if this heterodimer does not need RA in the above transactivation, RA may influence the transactivation of the PBRU by reducing the ligand-free RXR available for CAR-RXR formation. In this report, we focused our interests on the effect of CAR and RXR ligands in PB-mediated induction, and assessed this using a primary culture of rat hepatocytes.

MATERIALS AND METHODS

Materials. The following chemicals were purchased from the sources indicated: Sodium PB and 9-*cis*-RA (Wako Chemical Indus-

Abbreviations used: P450, cytochrome P450; PB, phenobarbital; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; RA, retinoic acid; AES, 5α-androsten-3α-ol; CAR, constitutive androstane receptor; RXR, retinoid X-receptor; PBRU, phenobarbital-responsive unit; PBREM, phenobarbital-responsive enhancer module; RARE, retinoic acid-responsive element; DR, direct repeat.

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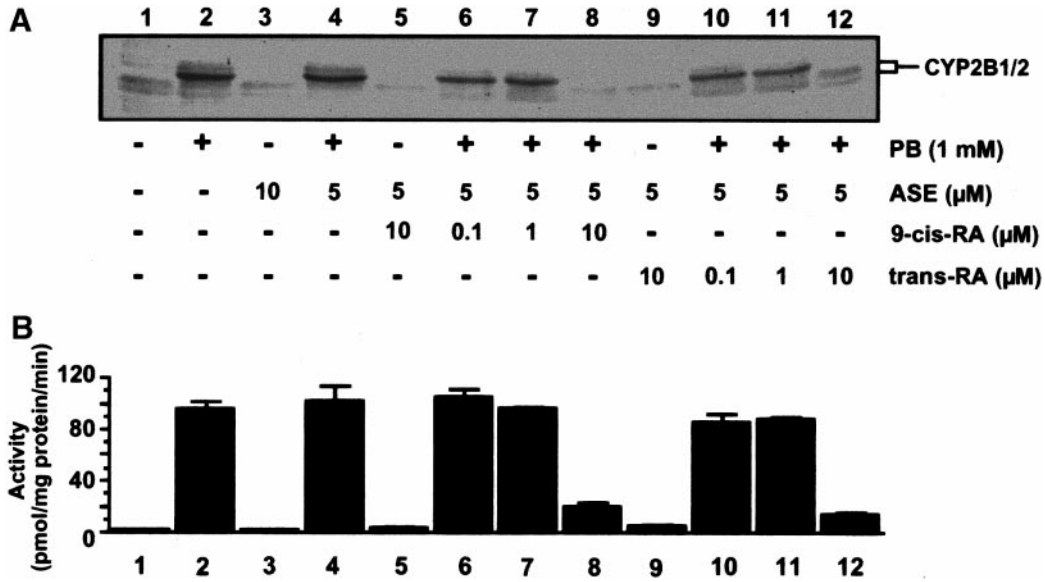

FIG. 1. The effects of ASE, and 9-*cis*- and all-*trans*-RA on the PB-mediated induction of CYP2B1/2 in cultured rat hepatocytes. The cells were treated with materials at the concentrations indicated in the figure for 2 days. Immunoblot analysis with anti-CYP2B1/2 antibody (A) and change in testosterone 16β-hydroxylase activity (B) are shown. In B, each bar represents the mean ± SE of three determinations. A representative result is shown, and the same data were obtained from two additional experiments in which the hepatocytes were prepared from different rats.

tries, Co. Ltd., Osaka, Japan); and ASE, all-*trans*-RA, 9-*cis*-retinal, all-*trans*-retinol and all-*trans*-retinal (Sigma Chemical Co., St. Louis, MO). 16β-Hydroxytestosterone was kindly donated by Dr. T. Baba, Shionogi Pharmaceutical Co., Osaka, Japan. Reagents for hepatocyte culture were obtained from commercial sources: Collagenase (Wako Pure Chemical Industries); Waymouth's MB 752/1 medium and fetal bovine serum (Gibco BRL, Grand Island, NY); and Matrigel (Becton Dickinson Labware Co., Bedford, MA). Rabbit anti-CYP2B1/2 antibody was prepared in this laboratory (15).

Hepatocyte culture. Male Sprague Dawley rats weighing 200–300 g were obtained from Charles River Japan (Kawasaki, Japan). Primary culturing of rat hepatocytes was performed essentially by a method described elsewhere (16). Briefly, isolated hepatocytes were prepared by *in situ* perfusion of liver with serum-free Waymouth MB-752 medium containing collagenase. The cells were washed, and seeded onto dishes precoated with Matrigel at 1×10^7 cells/dish. The cells were cultured for the first 24 h in medium containing bovine fetal serum, and then maintained in serum-free medium. Following the pre-culture for three days, the cells were treated with the test materials for two days. Hepatocyte microsomes were prepared by the reported methods (16).

Analytical methods. Sodium dodecylsulfate polyacrylamide gel (9%) electrophoresis (17) and the subsequent immunoblot analysis (18, 19) were carried out by the established methods. In all immunoblot analyses, 45 μg microsomal protein samples were electrophoresed and blotted. Testosterone 16β-hydroxylase activity, a marker for CYP2B1/2, was assayed by previously reported methods (20). The contents of protein (21) and total P450 (22) in hepatocyte microsomes were determined by the methods indicated.

RESULTS

The effects of ASE, and 9-*cis*- and all-*trans*-RA on the PB-mediated induction of CYP2B1 and 2B2 were assessed in a hepatocyte culture system, by monitoring the changes in immunoreactive CYP2B1/2 protein (Fig. 1A) and testosterone 16β-hydroxylase activity (Fig. 1B). Analyses of both parameters indicated that CYP2B1/2 increased markedly following treatment of the cells with 1 mM PB, under the culture conditions employed in this study. ASE alone (10 μM) showed no inductive effect. When combined with PB, ASE exhibited no suppression of PB-mediated induction. The effect of ASE on PB-mediated induction was assessed five times, using different preparations of hepatocytes. Although all data are not shown, the results showed that 5 μM ASE caused a 1.62 (± 0.56)-fold increase in testosterone 16β-hydroxylase activity vs. treatment with PB alone. Therefore, this study revealed an enhancing, rather than a suppressing, effect of ASE on PB-mediated induction of CYP2B1/2, in rat primary hepatocytes.

Interestingly, both 9-*cis*- and all-*trans*-RA reduced markedly the inductive effect caused by PB and ASE at a concentration of 10 μM, although lower concentrations (1 and 0.1 μM) of these retinoids had almost no effect (Figs. 1A and 1B). The content of total P450 in the cells co-treated with PB, ASE and 10 μM RAs was maintained at over the level of untreated cells (45.4 pmol/mg protein); for example, the P450 content of the hepatocytes treated with 10 μM 9-*cis*-RA, 10 μM ASE and 1 mM PB maintained to be 1.4-fold higher than that of untreated cells (data not shown). From these observations, the reduction of CYP2B1/2 level by RAs was assumed not to be due to cell damage. A similar suppression by RAs was also observed on PB-mediated

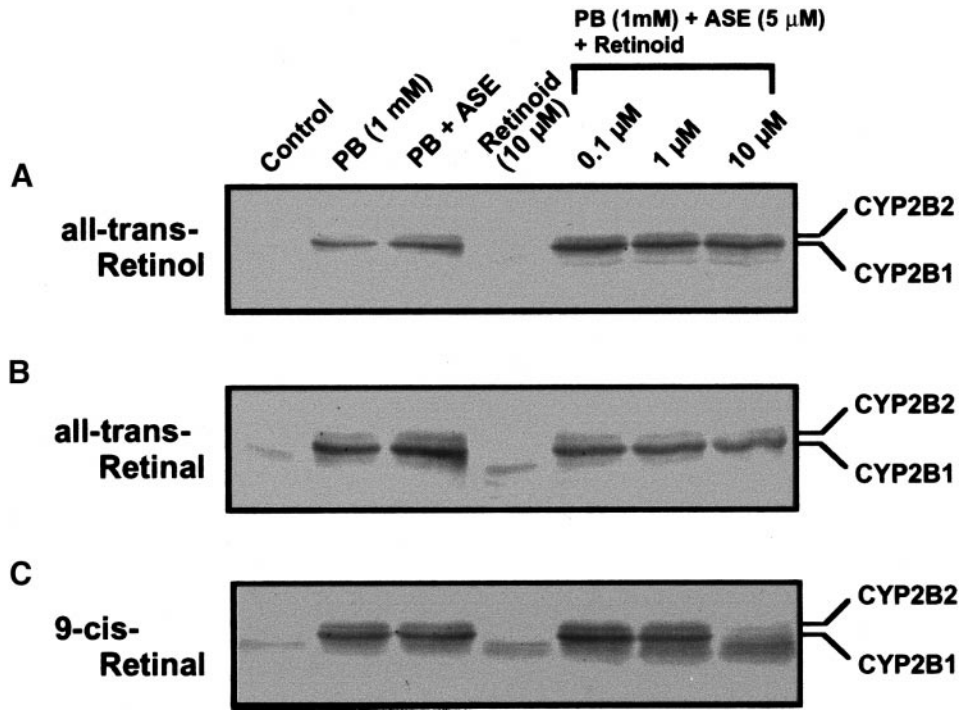

FIG. 2. The effects of all-*trans*-retinol (A) and -retinal (B), and 9-*cis*-retinal (C) on the PB-mediated induction of CYP2B1/2 in cultured rat hepatocytes. The cells were treated for 2 days with retinol/retinals or with these retinoids plus 1 mM PB and 5 μM ASE. The concentrations of retinoids added are indicated in the figure. Immunoblot analysis with anti-CYP2B1/2 antibody is shown. A representative result is shown, and the same data were obtained from two additional experiments in which the hepatocytes were prepared from different rats.

induction in the absence of ASE (data not shown). To further examine the specificity of RA, the effect of RA precursors was assessed. The results showed that 10 μM 9-*cis*-retinal suppresses the effect of PB, similar to the RAs, whereas all-*trans*-retinol and -retinal have no effect (Fig. 2). The dose–effect relationship was determined for the suppression produced by RAs and 9-cis-retinal (Fig. 3). Both the immunoblot analysis and testosterone 16β-hydroxylase assay indicated that 9-*cis*-RA and -retinal almost cancel the increase in CYP2B1/2 proteins caused by PB and ASE at a concentration of 3 μM. On the other hand, all-*trans*-RA needed higher concentrations to produce suppression than the above two compounds. Therefore, the suppression by 9-cis-RA/retinal on PB-mediated induction of CYP2B1/2 appears to be stronger than that by all-*trans*-isomers.

DISCUSSION

In this study, ASE produced no suppression of the PB-mediated induction of CYP2B1/2. Removal of ASE-dependent suppression of *CYP2B1/2* gene transactivation by TCPOBOP (7) seems reasonable, since the affinity of the latter compound for the CAR has been reported to be much higher than that of the former (10). However, PB has been suggested not to be a CAR agonist and to exert its inductive effect by mechanisms which differ from TCPOBOP (10). Early work that showed the absence of a high affinity binding site for ^{3}H-PB in rat hepatic subcellular fractions supports this view (23). Perhaps, the role of PB in CYP2B1/2 induction is, at least, facilitation of the translocation of the CAR from cytosol to nucleus, a process which may require protein phosphorylation and dephosphorylation (8, 24). A previous study demonstrating that PB induction does not need *de novo* protein synthesis (25) seems to agree with above mechanism. Since ASE appears to be an inverse agonist capable of suppressing gene transactivation (7–9), it is expected that exogenous ASE added to the hepatocytes suppresses the transactivating activity of the CAR enhanced in the nucleus by PB. However, this was not observed in the present study in cultured rat hepatocytes. The results presented here do not support the view that ASE plays a key role in suppression of *CYP2B1/2* gene expression *in vivo*. The mechanism by which ASE enhanced PB-mediated CYP2B1/2 expression to some extent remains unknown. It may be that the metabolite(s) formed from ASE act as a positive co-regulator for the CAR. A similar argument has been proposed in a recent review (3).

The CAR-RXR dimer has been suggested not to require RA for its transactivating function (11, 12). In

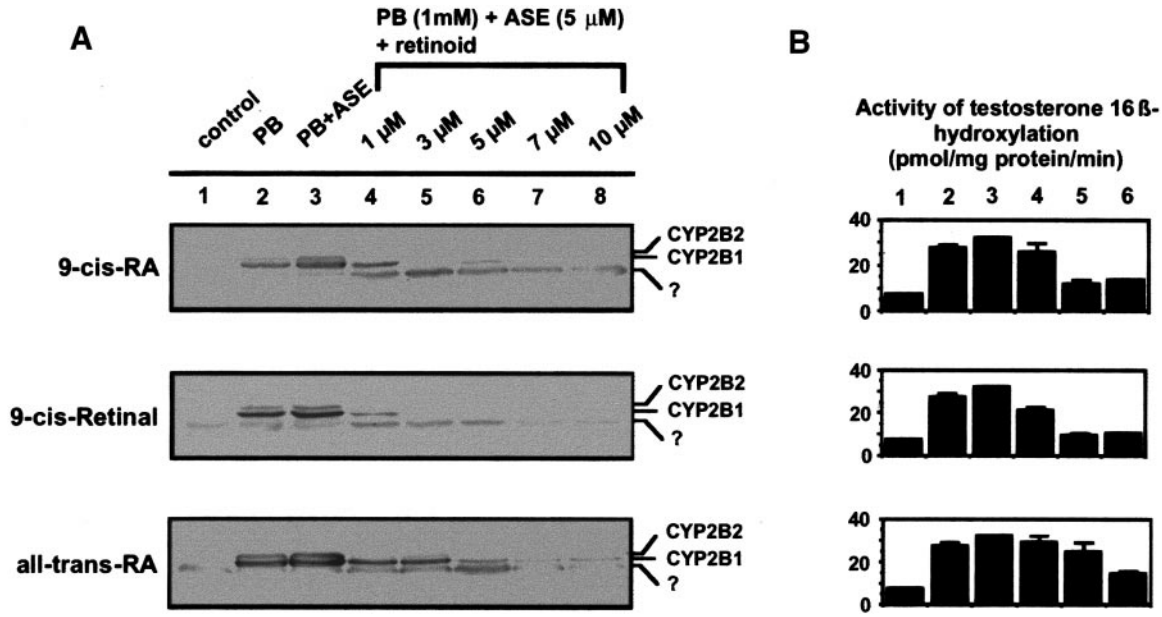

FIG. 3. The dose–effect relationship for the suppression of the PB-mediated induction of CYP2B1/2 by 9-*cis*-RA and -retinal, and all-*trans*-RA. The cells were treated for 2 days with retinal/RAs, 1 mM PB and 5 μM ASE. The concentrations of retinoids added are indicated in the figure. Immunoblot analysis with anti-CYP2B1/2 antibody (A) and change in testosterone 16β-hydroxylase activity (B) are shown. The activities in B are the mean ± SE of three determinations. The sample numbers shown in B correspond to those shown in A. A representative result is shown, and the same data were obtained from two additional experiments in which the hepatocytes were prepared from different rats.

contrast, this study showed that RAs suppress PB-mediated induction of CYP2B1/2. Taking both the non-requirement of RA for the CAR/RXR function and the suppression ability of RAs into consideration, we propose that excess RAs occupy the RXR and present it to signal transducing systems other than CAR/RXR, resulting in a reduction in the RXR available for the CAR. In clear contrast to the CAR, the RXR level in the hepatic nucleus remains unchanged before and after treating animals with inducers (8). Therefore, regulation of the RXR ligand supply to nucleus may be an important mechanism which affects CAR/RXR function. Concerning this issue, the present study showed the absence of an effect for all-*trans*-retinal and -retinol, although all-*trans*-RA produced positive suppression. From this observation, it appears that enzymes responsible for the conversion of precursors to 9-*cis*-RA play an important role. We failed to detect any suppression by all-*trans*-retinol at a concentration of 10 μM. The concentration of unesterified retinols in rat liver has been reported to be approximately 5 μM (26), which is near the concentration we employed, and so these data again support the view that the enzymatic ability to produce RA, but not the concentration of the precursors, is an important determinant of the regulation of nuclear transactivator complexes involving the RXR. Hepatic levels of retinols and retinyl esters have been reported to remain unchanged during the course of CYP2B induction (27). This observation also agrees with the above assumption. In this study, the maximal suppression of CYP2B1/2 expression was attained by adding 10 μM all-*trans*-RA to the medium. On the other hand, serum and hepatic contents of all-*trans*-RA in rats were reported to be approximately 2 nM (1.8 pmol/g tissue) and 11 nM (11.3 pmol/g tissue), respectively (28). This report also described that the content of 9-*cis*-RA in these tissues was much lower than that of all-*trans*-form. Thus, the concentration of exogenous RA needed for CYP2B1/2 suppression was far higher than the serum and cellular contents. Lansink *et al.* (29) suggested that the reason for the requirement of high concentration of RA for inducing gene regulation in cultured human cells including hepatocytes is rapid loss of RA due to the metabolism in the cells. The same may be true for RA-induced suppression of CYP2B1/2 shown in this study. It is known that RA taken up into liver distributes rapidly to rough endoplasmic reticulum as well as to nucleus (30). This observation seems to support a view that major portion of RA moved into liver is metabolized/inactivated by P450 (29). The relative roles of oxidase/dehydrogenases, P450 enzymes, and retinoid binding proteins involved in the formation and localization of RA are not fully understood (31–33), and need further clarification.

Quite recently, it has been reported that a number of compounds, including 9-*cis*-RA, are ligands for the PXR (34), a nuclear receptor belonging to the same subfamily (the NR1I subfamily) (35) as the CAR. From this observation, another mechanism seems possible and is worthy of examination, namely that RA may

produce suppression of PB-mediated induction by interacting with the CAR. However, since this study showed the superiority of 9-*cis*-RA over all-*trans*-RA, as far as suppression is concerned, we assume that RXR is involved. The reason that all-*trans*-RA exhibited an effect is likely to be due to the metabolic isomerization to 9-*cis*-RA of the all-*trans*-form (36). The suppression with 9-*cis*-retinal was almost equivalent to 9-*cis*-RA (Fig. 3), and this effect might be due to 9-*cis*-RA metabolically formed in the cells.

To our knowledge, the effect of feeding rats an RA-deficient diet on PB-mediated induction has not been studied. However, rats fed a vitamin A-deficient diet did not show any significant change in the hepatic activity of testosterone 16β-hydroxylase (37). This data suggests that removal of RA-dependent suppression is insufficient for enhanced expression of CYP2B1/2 and a concomitant event distinct from this suppression, such as CAR translocation or activation, is needed. Howell *et al.* (38) have reported enhancement of CYP2B1/2 expression by RXR ligands, including 9-*cis*-RA, by testing them *in vivo.* However, since this study confirmed no such inductive effect of 9-*cis*-RA in cultured hepatocytes, the inductive effect of RA observed *in vivo* seems to be outcome due to an indirect effect of RA. One possibility is that the *in vivo* inductive effect of RA might be due to a change in the levels of circulating hormones. A previous observation may support the view that the secretion and the pattern of growth hormones, one of the regulators of the CYP2B subfamily (39), is influenced by RA (40, 41).

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