EFFECT OF A LIGAND SELECTIVE FOR PERIPHERAL BENZODIAZEPINE RECEPTORS ON THE EXPRESSION OF RAT HEPATIC P-450 CYTOCHROMES: ASSESSMENT OF THE EFFECT IN VIVO AND IN A HEPATOCYTE CULTURE SYSTEM

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(Received February 1, 1999; accepted June 28, 1999)

This paper is available online at http://www.dmd.org

ABSTRACT:

The peripheral benzodiazepine receptor plays a role in the translocation of cholesterol into mitochondria where steroidogenesis occurs. Sterols have been suggested to be involved in the regulation of the cytochrome P-450 (CYP)2B subfamily as the endogenous suppressor of this CYP. To investigate the role of cholesterol metabolites on the expression of CYPs, the effect of PK11195, a specific ligand of the peripheral benzodiazepine receptor and a stimulator of cholesterol transportation, on CYP expression was examined in rats in vivo and in cultured hepatocytes. As judged by the change in testosterone metabolic activity catalyzed by liver microsomes, i.e. injection of PK11195 into rats increased the CYP2B subfamily significantly. A trend in the induction of the CYP2A1, 2C11, and 3A isozymes was also observed. When PK11195 was given to rats together with phenobarbital, an additive effect of these compounds on testosterone metabolic activity was observed. In cultured hepatocytes, PK11195 exhibited the same effect on CYP expression as seen in vivo, but the magnitude of the effect was much greater than that observed in vivo. The inductive effect of PK11195 toward the CYP2B and 3A subfamilies was 2.3- and 6.5-fold greater, respectively, than that with phenobarbital. The inductive effect of PK11195 was confirmed by immunoblotting with antibodies against CYP2A, 2B, 2C, and 3A proteins. These results indicate that PK11195 has an inductive effect on several subfamilies of CYPs by directly acting on liver cells and has no ability to suppress the expression of these CYPs. This observation suggests that, if certain sterols play a role in the suppressive control of the CYP2B subfamily, they are produced in organelles other than the mitochondria.

The mechanism by which phenobarbital (PB) induces the hepatic cytochrome P-450 (CYP) 2B subfamily is largely unknown, although the interaction between specific regions of the gene upstream and the trans-acting factor has been studied by many workers (He and Fulco, 1991; Ransdell et al., 1993; Shepherd et al., 1994; Prabhu et al., 1995; Trotter et al., 1995; Honkakoski et al., 1998). In particular, the target with which PB interacts, reflecting the increase in CYP2B protein, has not been identified at all. One hypothesis suggests that the constitutive form of CYP is the target of PB (Waxman and Azaroff, 1992). In this hypothesis, xenobiotic inducers of the CYP2B subfamily are inhibitors of the constitutive form that metabolizes endogenous substance to activate repressor or inactive inducer participating in the CYP2B induction. On the other hand, Kocarek et al. (1993) have reported that mevalonol, a hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitor induces the hepatic CYP2B subfamily in primary culture cells from rat liver. This observation demonstrates the contribution of steroid(s) to the down-regulation of the CYP2B subfamily. The same has been suggested by a study using squalene synthase (Kocarek et al., 1998). As far as this is concerned, 25-hydroxycholesterol has been found to reduce the CYP2B expression level in PB-treated rat hepatocytes (Kocarek et al., 1993). It is, therefore, conceivable that PB inhibits the constitutive form of CYP, which plays a role in producing cholesterol metabolite(s), and this is a key event in the induction of the CYP2B subfamily. However, this possibility remains to be clarified.

The peripheral benzodiazepine receptor (PBR) plays an important role in steroidogenesis by translocating cytosolic cholesterol into mitochondria (Papadopoulos and Brown, 1995). PK11195 (Fig. 1) is a high-affinity ligand of PBR (Hirsch et al., 1989; Sprengel et al., 1989; Papadopoulos et al., 1990), and has been shown to facilitate the translocation of cholesterol into mitochondria in liver cells as well as in steroidogenic cells (Papadopoulos et al., 1990; Tsankova et al., 1995). In this study, we examined the effect of PK11195 on the induction of rat hepatic CYP2B1/2 to see whether cholesterol metabolites produced in mitochondria contribute to the induction. It is possible that PK11195 affects the magnitude of CYP2B1/2 induction through a change in the endocrine level of steroid hormones by acting on steroidogenic tissues. To avoid this possibility, we estimated the effect of PK11195 in a hepatocyte primary culture system as well as in vivo.

Experimental Procedures

Materials. PK11195 was purchased from Sigma Chemical Co. (St. Louis, MO). Hydroxytestosterone standards were donated by Dr. T. Baba, Shionogi Pharmaceutical Co. (Osaka, Japan). Polyclonal rabbit antibodies raised against purified CYP2B1, CYP2A1, and CYP2C11 were prepared by our own laboratory. The specificity of the former two antibodies has been reported elsewhere (Nagata et al., 1985; Yamada et al., 1996). The antibody against
CYP2C11 has not been characterized in detail, although this antibody is able to recognize purified CYP2C11 (H.Y., T. Baba, K.O. and H. Yoshimura, unpublished data). Anti-CYP3A2 antibody was obtained from Daichi Pure Chemicals Co. (Tokyo, Japan). Alkaline phosphatase-conjugated goat anti-rabbit IgG antibody was purchased from Zymed Laboratories, Inc. (San Francisco, CA). The following materials for hepatocyte culture were purchased from the sources indicated: collagenase (Wako Pure Chemical Industries, Osaka, Japan), Matrigel (Becton Dickinson Labware Co., Bedford, MA), Waymouth’s MB 752/1 medium and bovine fetal serum (Gibco BRL, Grand Island, NY), and bovine pancreas insulin (Sigma). Modified Waymouth’s MB 752/1 medium was prepared by adding the following materials: 26.7 mM NaHCO₃, 18 mM HEPES, 23.8 mM Na₂SeO₃, 1 × 10⁻² M zinc sulfate, 100 mg/liter streptomycin sulfate, 250 μg/liter fungizone, and 6.25 mg/liter insulin. The pH of the medium was adjusted to 7.2 with 1 N NaOH.

**Animals and Preparation of Liver Microsomes.** Male Wistar rats weighing about 150 g were treated i.v. with sodium heparin at a dose of 10 mg/ml saline/kg, and anesthetized with halothane. The livers were then removed. Liver microsomes were prepared by the methods established method (Omura and Sato, 1964). Statistical differences were estimated by the method of Laemmli (1970). The proteins in the gel were stained with Coomassie blue R-250. EF. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and incubated with the primary antibodies. The bands reacting with the antibody were visualized by the method of Blake et al. (1984) using alkaline phosphatase-conjugated anti-rabbit IgG antibody. Testosterone-metabolizing activity was assayed by the method described elsewhere (Shinohara et al., 1997). Briefly, testosterone was incubated with microsomes supplemented with NADPH, and the metabolites were extracted with ethyl acetate followed by analysis using reversed phase HPLC. In the assay of the microsomal activity of cultured hepatocytes, 0.5 mM testosterone was incubated with 0.3 mg microsomal protein according to the method of Murayama et al. (1996). The content of total CYP was determined by an established method (Omura and Sato, 1964). Statistical differences were estimated by ANOVA with a posthoc test (Fisher’s Protected Least Significant Difference method).

**Results**

Chronic i.p. injection of PK11195 (5 mg/kg/day × 4) to rats significantly increased the hepatic content of total CYP, but the magnitude of this increase was much less than that with PB, a reference inducer (Table 1, experiment B). When PK11195 was given to rats together with PB, an additive effect was observed (Table 1, experiment B).

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CYP Content Relative to Control</th>
<th>Relative to PB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (N = 3)</td>
<td>1.29 ± 0.27</td>
<td>1</td>
</tr>
<tr>
<td>PB × 1 (N = 3)</td>
<td>1.97 ± 0.04*</td>
<td>1.53</td>
</tr>
<tr>
<td>PK11195 (5 mg/kg) × 1 (N = 3)</td>
<td>1.55 ± 0.19</td>
<td>1.20</td>
</tr>
<tr>
<td>[PB + PK11195 (5 mg/kg)] × 1 (N = 4)</td>
<td>2.11 ± 0.32*</td>
<td>1.63</td>
</tr>
<tr>
<td>[PB + PK11195 (25 mg/kg)] × 1 (N = 3)</td>
<td>2.18 ± 0.13*</td>
<td>1.69</td>
</tr>
<tr>
<td>Experiment B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (N = 3)</td>
<td>1.45 ± 0.06</td>
<td>1</td>
</tr>
<tr>
<td>PB × 4 (N = 3)</td>
<td>2.83 ± 0.33*</td>
<td>1.95</td>
</tr>
<tr>
<td>PK11195 (5 mg/kg) × 4 (N = 3)</td>
<td>1.67 ± 0.10*</td>
<td>1.15</td>
</tr>
<tr>
<td>[PB + PK11195 (5 mg/kg)] × 4 (N = 3)</td>
<td>3.83 ± 0.18*</td>
<td>2.64</td>
</tr>
<tr>
<td>[PB + PK11195 (25 mg/kg)] × 4 (N = 4)</td>
<td>3.76 ± 0.16*</td>
<td>2.59</td>
</tr>
</tbody>
</table>

In Experiment A, PB (80 mg/kg) and/or PK11195 (the dose indicated in the table) were given once to rats. In Experiment B, PB and/or PK11195 were given to rats for 4 consecutive days.

* Significantly different from the control (P < 0.05).

§ Significantly different from the PB-treated group (P < 0.05).
PK11195 and PB increased 6 although a trend toward an increase was seen. Double treatment with antagonize the effect of PB.

potent inducer of several forms of hepatic CYP, and is unable to experiment (Fig. 2). These results clearly indicated that PK11195 is a 7 and 5.7-fold less than those produced at the low concentration. Only significantly increased but the magnitudes of the increase were 5.0- and 60-fold over the control by 10 μM PK11195. This effect was 2.3-fold greater than that of PB. Except for 7α-hydroxylation, all the activities were enhanced by PK11195 (10 μM) more markedly than by PB. In particular, the effects of the two compounds on 2β- and 6β-hydroxylation were very different, and this activity was increased specifically by PK11195. Cotreatment of the hepatocytes with PB and 10 μM PK11195 resulted in an additive or synergic effect on the increase in 2β-, 6β-, and 7α-hydroxylase activity in accordance with the in vivo experiment (Fig. 2). These results clearly indicated that PK11195 is a potent inducer of several forms of hepatic CYP, and is unable to antagonize the effect of PB.

A higher concentration (100 μM) of PK11195 exhibited very different effects from the low concentration as far as testosterone-metabolizing activity was concerned; namely, 2α- and 16α-hydroxylation and 17-oxidation were significantly reduced compared with the control by 100 μM PK11195, and 6β- and 16β-hydroxylation were significantly increased. The magnitudes of the increase were 50- and 5.7-fold less than those produced at the low concentration. Only 7α-hydroxylation was increased more markedly by 100 μM than by 10 μM. When the hepatocytes were cotreated with PB and 100 μM PK11195, all metabolic activity was greatly reduced in comparison with that obtained with PB + 10 μM PK11195. The reason that the high concentration of PK11195 abolishes most of the activity of testosterone metabolism, whereas the low concentration increases it, remains unclear. It is, however, considered likely that the nonspecific suppression of CYPs by a high concentration of PK11195 is due to the toxicity of this compound (see Discussion for more details).

Immunoblot analyses (Fig. 4) agreed well with the change in testosterone metabolic activity. For example, supporting the change in testosterone 16β-hydroxylase activity mentioned above, CYP2B1/2 was increased more markedly in 10 μM PK11195-treated hepatocytes than in PB-treated cells. In the cells cotreated with PB and 10 μM PK11195, comparable CYP2B1/2 bands to those seen in the cells treated with the latter compound alone were observed. The higher concentration of PK11195 greatly reduced the amounts of both isozymes. In Fig. 4A, the separation of CYP2B1 and 2B2 bands was unsatisfactory. However, they were clearly distinguished in another experiment (Fig. 4D) in which anti-CYP2A1 antibody was used. This antibody recognized purified CYP2B1/2 as well as purified CYP2A1 (data not shown). From this immunoblot, both CYP2B1 and 2B2 were confirmed to be increased by 10 μM PK11195. PB- and PK11195-caused increases in CYP2A1 were also detected (Fig. 4D). This was virtually matched by the change in testosterone 7α-hydroxylase activity. However, it should be noted that the high concentration of PK11195 exhibited a weaker immunoblot band for CYP2A1 than did the low concentration, whereas the reverse was observed for the change in 7α-hydroxylase activity (Fig. 3). The reason for this discrepancy is not clear. In accordance with the alteration in 2β- and 6β-hydroxylase activity, the CYP3A isozyme(s) was greatly increased by 10 μM but not by 100 μM PK11195 (Fig. 4B). The band intensity was greater in the hepatocytes treated with PB plus PK11195 than with PK11195 alone. The expression of CYP2C11 was also increased by 10 μM PK11195, and the effect was a little greater than that of PB (Fig. 4C). This observation was consistent with the change in testosterone 2α-/16α-hydroxylase activity. However, it should be noted that although cotreatment with PB and 10 μM PK11195 caused more pronounced 2α-/16α-hydroxylase activity than did single treatments, the band intensity of CYP2C11 in the immunoblot was stronger in cells treated with PK11195 than in cotreated cells.

Discussion

The study in cultured rat hepatocytes clearly indicates that PK11195, a specific ligand of the PBR, is an inducer of CYP2A1, 2B1/2, 2C11, and 3A isozymes, and suggests that this compound has no suppressive effects on the expression of these CYPs and PB-
mediated induction. The reason that the high concentration of PK11195 caused nonspecific reduction in CYP isozymes remains unclear. It is, however, considered likely that the suppression of CYPs with a high concentration of PK11195 is due to the toxicity of this compound. The observation that PB treatment together with a high concentration of PK11195 causes more marked reduction in CYPs has a dose-dependent biphasic effect on the expression of CYPs. However, as can be seen in Fig. 4D, immunoblot analysis of CYP2A1 did not match the change in testosterone 7α-hydroxylase activity; although the 7α-hydroxylase activity was increased by 100 μM PK11195, the CYP2A1 immunoblot band was reduced by this treatment. This inconsistency suggests that the increase in testosterone 7α-hydroxylase activity with 100 μM PK11195 was overestimated from some unknown reason. If this was the case, it would be reasonable to believe that a high concentration of PK11195 damages cultured hepatocytes.

The inducing effect of PK11195 differed in magnitude between the in vivo and cultured hepatocyte experiments. Although a minor increase, less than 2-fold, in the activity of testosterone 6β- and 16β-hydroxylases was observed in vivo, a marked increase in this activity was seen with PK11195 in cultured hepatocytes. Although the reason for this inconsistency was not clear, the low increase in the in vivo experiment is probably due to the low dose used in this study. An early report showed that PK11195 is rapidly metabolized in the body, although no details of its metabolic fate were given (Totis et al., 1989). In support of this, when PK11195 is given orally, rats tolerate high doses, e.g., 500 mg/kg (Totis et al., 1989; Strazielle et al., 1991), which are much higher than the dose used in this study (5 or 25 mg/kg i.p.). The above workers have reported that a high oral dose of PK11195 causes induction of the CYP2B and 3A proteins and mR-
NAs, and the effects are comparable with those seen with PB. It is, therefore, likely that the maximal biological effect of PK11195, at least on CYP expression, is obtained after oral administration of the high dose, but not by parenteral routes. As mentioned already, higher i.p. doses were not available because of their toxicity.

The in vivo inductive effect of PK11195 on the CYP2B subfamily was weaker than PB (this study), or comparable with PB (Strazielle et al., 1991). However, in cultured hepatocytes, the effect of PK11195 was much greater than PB. Furthermore, as judged by the change in testosterone 6β-hydroxylase activity, the inductive effect of PK11195 on CYP3A in primary culture was about 7-fold greater than with PB whereas, in vivo, the effect was comparable with PB. This contradiction seems again to be due to an insufficient amount of PK11195 targeted to the liver when administered in vivo. However, because PK11195 stimulates intermembrane translocation of cholesterol in mitochondria and enhances steroidogenesis (Papadopoulos et al., 1990; Tsankova et al., 1995), there is another possibility, i.e., that the in vivo inductive effect of PK11195 is partially canceled by steroid hormone(s) the circulating levels of which are increased by PK11195 treatment.

Porphyrias are suggested to be one of the endogenous ligands for the PBR (Verma et al., 1987; Snyder et al., 1987), and this receptor is considered to play a role in the translocation of not only cholesterol but also coproporphyrinogen, a precursor in heme synthesis (Takataki et al., 1995). PK11195 competes with porphyrins at the PBR binding site and inhibits the production of protoporphyrinogen (Takataki et al., 1995). Because the positive role of heme in the regulation of CYP expression has been demonstrated (Ravishankar and Padmanaban, 1986; Dwarki et al., 1987; Sultana et al., 1997), it is conceivable that the inhibitory effect of PK11195 on heme synthesis is different in vivo from that in culture, and this is the reason for the different responses of the two systems in CYP2B/3A expression against PK11195. However, there is a report that does not support this: Sinclair et al. (1990) have demonstrated that induction of CYP2B and 3A isoforms in cultured hepatocytes is independent of changes in heme synthesis.

Based on the observation that the inhibitors of HMG CoA reductase (Kocarek et al., 1993) and squalene synthase (Kocarek et al., 1998) are potent inducers of the rat CYP2B subfamily, certain steroid(s) are considered to be involved in the regulation of this CYP. In these studies, the effects of steroid biosynthesis inhibitors were examined in vivo and in cultured hepatocytes prepared by the same methods as in this study. The results support one of the possible mechanisms in which the expression of the CYP2B subfamily is under suppressive control by endogenous steroids; PB causes induction of this CYP by removing the negative control. Oxysterols, including 25-hydroxycholesterol, 27-hydroxycholesterol, 24,25-epoxycholesterol, and 25,26-epoxycholesterol, are suggested as candidates for the endogenous regulator (Kocarek et al., 1998). The inhibitory effect of 25-hydroxycholesterol on the induction of the CYP2B subfamily by PB, HMG CoA reductase inhibitor, and squalene synthase inhibitor seems to agree with the above view (Kocarek et al., 1993, 1998). However, besides 25-hydroxycholesterol, there may be another effector(s). This is supported by the following evidence: 1) 25-hydroxylation of cholesterol occurs in the mitochondrial fraction (Fredriksson, 1956; Björkhem and Gustafsson, 1974); and 2) PK11195, which is expected to increase the production of 25-hydroxycholesterol by enhancing cholesterol transportation into mitochondria, causes induction rather than suppression of the CYP2B subfamily (this study). The observation with PK11195 suggests that, if certain steroids play a role in the suppression of the CYP2B subfamily, they are produced in organelles other than mitochondria. An early work reported by Björkhem and Gustafsson (1973) might indicate a breakthrough in clarifying the induction mechanism of the CYP2B subfamily. They indicated that 26-hydroxylation of C27-steroids is catalyzed by microsomal enzymes as well as mitochondrial ones, and the microsomal, but not the mitochondrial, activity can be inhibited by PB. The role of this reaction in CYP2B induction is currently being investigated in our laboratory.

In conclusion, the present study suggests that PK11195, a selective ligand of the PBR, which can facilitate transportation of cholesterol into mitochondria, induces rat hepatic CYPs including the CYP2A2, 2B, 2C, and 3A subfamilies by acting directly on liver cells. PK11195 exhibits its maximal effect on CYP expression after oral administration but not parenterally, probably due to its rapid metabolism. This observation suggests that certain steroid(s) produced by nonmitochondrial enzymes play a role in the regulation of the CYP2B subfamily if involved in the mechanism.

Acknowledgments. We thank Dr. Y. Tanaka of our faculty, Dr. A. Toda, Daichi College of Pharmacy, Fukuoka, Japan, and Dr. M. Shimada, Tohoku University, Aoba, Japan, for their invaluable instruction regarding the experimental technique used for primary culture.

References


