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Effect of Phenobarbital on Intralobular Expression of CYP2B1/2 in Livers of Rats

DIFFERENCE IN THE EXPRESSION BETWEEN SINGLE AND REPETITIVE ADMINISTRATIONS

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ABSTRACT. Phenobarbital (PB) was shown to induce the major PB-inducible cytochrome P450 (CYP) isoforms, CYP2B1/2, in perivenular hepatocytes by a single injection, and in midzonal and periportal hepatocytes in addition to perivenular hepatocytes by injections of the same dosage once a day for 3 days in rat livers. The present study was undertaken to determine whether the spread of enzyme induction to midzonal and periportal hepatocytes is caused by the increase in total dose of the drug by repetitive injections or by the repetitive injections of the drug themselves. Male adult rats were administered PB by a single injection (80 mg/kg) or repetitive injections (20 mg/kg once a day for 4 days; a total dose of 80 mg/kg), and the molar content of CYP2B1/2 was measured by quantitative immunohistochemistry in the cytoplasm of perivenular, midzonal, and periportal hepatocytes. In addition, the molar content of total CYP in the cytoplasm was measured by microphotometry, and the expression of CYP2B2 mRNA was examined by *in situ* hybridization. When animals received the single injection, the isoforms and CYP2B2 mRNA increased markedly in perivenular hepatocytes, increased somewhat in midzonal hepatocytes, and remained unchanged in periportal hepatocytes. If animals received the repetitive injections, however, although the isoforms and the mRNA increased markedly in perivenular hepatocytes, they also increased markedly in midzonal hepatocytes and somewhat in periportal hepatocytes. These findings demonstrated that the enlargement of the sublobular area in which induction of the isoforms occurred was caused by the repetitive injections of PB themselves. BIOCHEM PHARMACOL **60**;2:285–291, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. CYP2B1/2; phenobarbital; quantitative immunohistochemistry; *in situ* hybridization; liver; rat

The major PB†-inducible CYP isoforms, CYP2B1/2, are localized restrictedly [1] or predominantly [2] in perivenular hepatocytes in normal rats. The isoforms are increased predominantly in perivenular hepatocytes by a single injection of 80 mg/kg of PB [2], and are increased markedly or moderately in midzonal or periportal hepatocytes in addition to perivenular hepatocytes by injections of PB (80 or 100 mg/kg) once a day for 3 days [1, 3]. Thus, PB appears to induce CYP2B1/2 isoforms by a single injection in perivenular hepatocytes, and by repetitive injections to enlarge the sublobular area in which induction of the isoforms occurs. However, it is unclear whether the enlargement of the sublobular area is caused by the increase in total dose of the drug by repetitive injections or by the repetitive injections of the drug themselves. To clarify this, PB was administered to rats at a total dose of 80 mg/kg body weight; one group of animals received a single injection (80 mg/kg), and the other group received repetitive injections (20 mg/kg once a day for 4 days). We measured the molar content of CYP2B1/2 by quantitative immunohistochemistry in the cytoplasm of perivenular, midzonal, and periportal hepatocytes. In addition, the molar content of total CYP in the cytoplasm was measured by microphotometry, and expression of CYP2B2 mRNA was examined by *in situ* hybridization.

MATERIALS AND METHODS

Fifty-four male Sprague–Dawley rats, 6- to 8-weeks-old (200–250 g, Nihon CLEA), were used. The animals were fed laboratory chow and water *ad lib.*, and were divided into three groups. One group of animals received a single i.p. injection of PB dissolved in saline at a dose of 80 mg/kg body weight. The second group received repetitive i.p. injections of PB dissolved in saline at a dose of 20 mg/kg body weight once a day for 4 days. For the control, animals received i.p. injections of saline once a day for 4 days. The animals were killed 24 hr after the last injection. The

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† *Abbreviations*: PB, phenobarbital; CYP, cytochrome P450; DIG, digoxigenin; ASI, average staining intensity; and SIP, staining intensity in portion.

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animal experiments were performed according to the guidelines of Kansai Medical University.

Immunohistochemical Methods

Under pentobarbital anesthesia, livers of animals (6 animals/group) were perfused with saline briefly, then perfused with cold 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min. For immunohistochemistry, animals injected singly with PB (80 mg/kg) and killed 4 days after the injection and those injected singly (160 mg/kg) and killed 1 day after the injection also were examined. Slices cut from the left lobe of the liver were washed with 0.1 M phosphate buffer (pH 7.4) containing 8% sucrose at 4° for 6 hr. Serial frozen sections, 6 μm in thickness, were cut and placed on poly-L-lysine-coated glass slides. The thicknesses of the slides and sections were checked as described previously [4, 5].

A pair of the serial sections (about 2 × 3 mm) were immersed in 70% (v/v) methanol containing 0.3% (v/v) hydrogen peroxide at room temperature for 30 min, washed with PBS (5 min x 3 times), and soaked in normal rabbit serum (diluted 1:10 with PBS) at room temperature for 30 min. Then, one section was incubated in PBS containing anti-rat CYP2B1/2 goat IgG (diluted 1:200 with PBS; Daiichi Kagaku), while the other was incubated with normal goat serum (diluted 1:50 with PBS) at 4° for 12 hr (80 μL/section). Two bands, corresponding to the molecular mass of CYP2B1/2, were detected around 50 kDa with the antibody as revealed by western blotting, and the antibody did not cross-react to other PB-inducible isoforms (CYP2C6 and 3A1/2) or non-PB-inducible isoforms (CYP1A1/2, 2E1, and 4A1) as revealed by dot western blotting (data not shown). After washing with PBS, the sections were incubated in PBS containing horseradish peroxidase-labeled rabbit anti-goat IgG (Cappel; diluted 1:200 with PBS) at 20° for 30 min. The above-mentioned incubation conditions appeared to follow saturation kinetics [2, 3]. The sections were washed again with PBS and stained with PBS containing 0.3 mg/mL of diaminobenzidine and 0.03% (v/v) hydrogen peroxide at 20° for 20 min. Finally, the sections were dehydrated and mounted.

Image Analysis of CYP2B1/2

The immunostained sections were subjected to image analysis with a microscope (TMD) linked to a video image processor (ARGUS 50, Hamamatsu Photonics). Microscopic images taken through an IF-470 interference filter (Nikon) were transferred to the processor. Average absorbances in widely defined areas of the sections (0.998 × 0.998 mm; average staining intensity, antibody-reacted; ASIab) and absorbances in small portions (3.9 × 3.9 μm; staining intensity in portion, antibody-reacted; SIPab) in the cytoplasm of periportal, midzonal, and perivenular hepatocytes were measured [4–6]. The average absorbance in a widely defined area (ASIns), and the absorbances of corresponding small portions in the section (SIPns) were also measured in the adjacent sections incubated with normal serum. The number of measurements per group was 1296 (2 portions/cell, 27 cells/slice, 2 slices/animal, and 6 animals/group; the same number of measurements were done on the adjacent sections incubated with normal serum instead of the primary antibody).

Microphotometry of Total CYP

Under pentobarbital anesthesia, livers of animals (6 animals/group) were perfused with cold saline for 15 min. Serial frozen sections, 20 μm in thickness, were cut from the left lobe of the liver, and the content of total CYP in the sections was measured microphotometrically as described previously [7–10]. In short, absorbances at 450 and 490 nm in reduced and carbon monoxide-bound spectra of CYP were measured in the cytoplasm of 9 periportal, midzonal, or perivenular hepatocytes with a spot size of 5 μm. The extinction of total CYP calculated from the absorbances was converted to the amount of total CYP per unit cytoplasmic volume. The number of measurements per group was 1296 (4 spots/cell, 27 cells/slice, 2 slices/liver, and 6 animals/group).

In Situ *Hybridization of CYP2B2 mRNA*

In situ hybridization was done as described previously [2]. In short, frozen sections, 6 μm in thickness, cut from livers of animals were placed on aminopropyltriethoxysilane-coated glass slides and treated with proteinase K followed by acetic anhydride. Then, the sections were hybridized with a DIG-labeled oligonucleotide probe for CYP2B2 mRNA (100 pmol/mL) in diluted sodium citrate–sodium chloride solution (5x SSC, 0.75 M NaCl and 75 mM sodium citrate) containing 2% (w/v) DIG Blocking Reagent™ (Boehringer Mannheim), 50 mM sodium phosphate, 7% (w/v) SDS, 0.1% (v/v) *N*-laurylsarcosine, 200 μg/mL of tRNA, and 50% (v/v) formamide (pH 7.0). The antisense sequence of the probe was 3′-CACTAACCGAGAGTGTC-CGGTGGTAGGGAA-5′ for CYP2B2, corresponding to amino acids 331 through 340 [2]. After hybridization, the sections were washed with 5x SSC and stained with a DIG Detection System Kit™ (Boehringer Mannheim).

Biochemical Methods

The remaining liver tissues prepared for the measurement of total CYP by microphotometry were homogenized with 9 vol. of PBS containing 0.25 M sucrose. CYP2B1/2 content in the homogenate was measured by single radial immunodiffusion [8, 11]. Total CYP content in the homogenate was analyzed by difference spectrophotometry [4, 10]. Protein content was measured by the method of Dulley and Grieve [12].

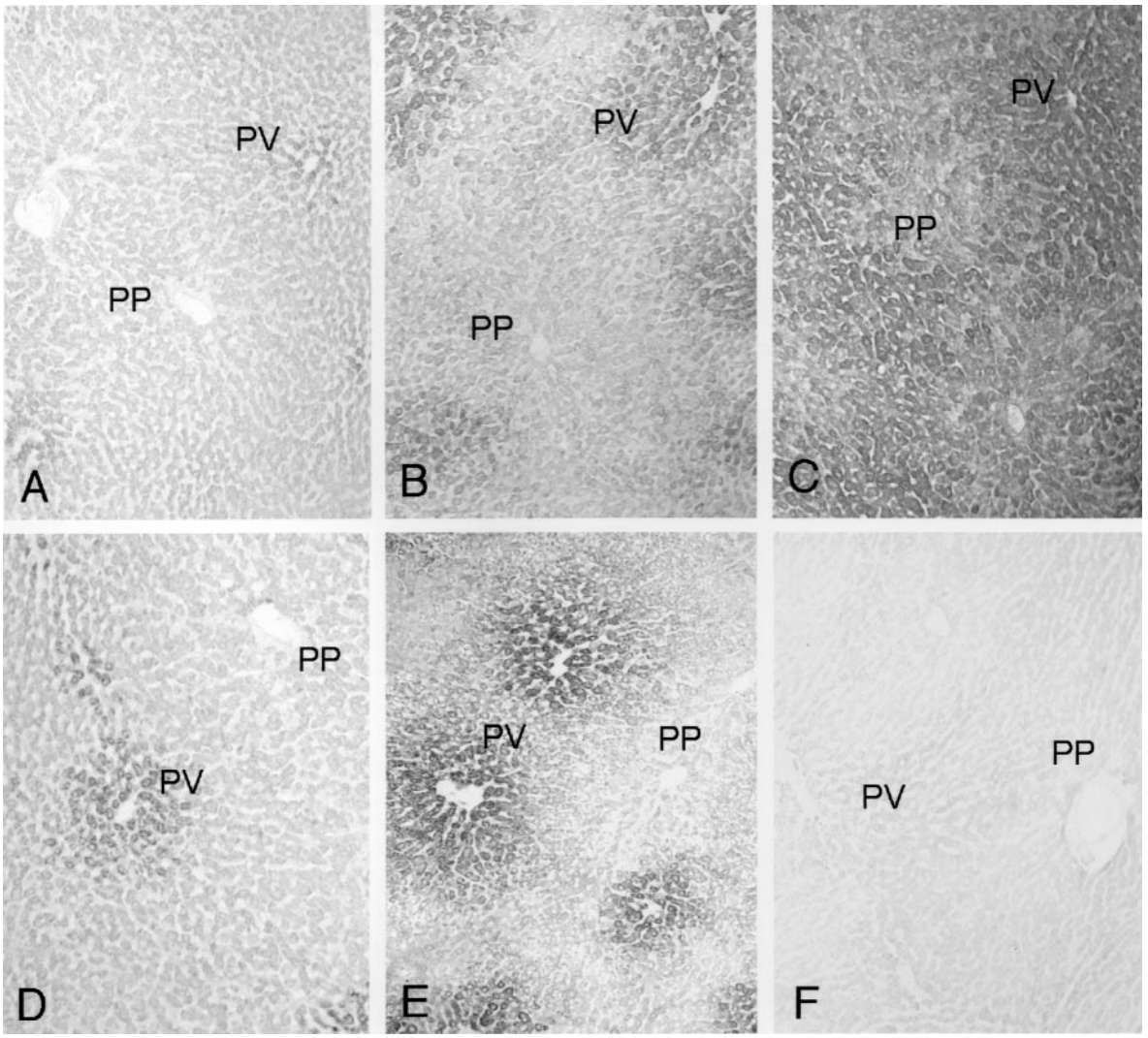

FIG. 1. Immunostaining of CYP2B1/2 isoforms detected by the indirect immunoperoxidase method under saturation conditions in frozen sections cut from rat livers perfused with buffered 4% formaldehyde. Sections were incubated with anti-CYP2B1/2 antibody (A–E) or normal serum instead of the antibody (F). (A) Control rat; (B) animal injected singly with PB at a dose of 80 mg/kg body weight and killed 1 day after the injection; (C and F) animal injected repetitively with PB at a dose of 20 mg/kg body weight once a day for 4 days and killed 1 day after the last injection; (D) animal injected singly with PB at a dose of 80 mg/kg body weight and killed 4 days after the injection; (E) animal injected singly with PB at a dose of 160 mg/kg body weight and killed 1 day after the injection. PP, periportal area; PV, perivenular area. Magnifications, ×50.

Statistical Analysis

Data were analyzed by the Bartlett test and then subjected to one-way ANOVA followed by Duncan's multiple-range test. All statistical comparisons were made above the 95% level of confidence.

RESULTS

Immunostaining of CYP2B1/2 isoforms detected under saturation conditions was seen in the cytoplasm of hepatocytes in livers of control and PB-treated rats (Fig. 1, A–E), whereas the staining was negligible in sinusoidal cells and the nuclei of hepatocytes. In control animals, the staining was weak in perivenular hepatocytes, and very weak in midzonal and periportal hepatocytes (Fig. 1A). In livers of animals administered PB with a single injection (80 mg/kg × 1), the staining was strong in perivenular hepatocytes, weak in midzonal hepatocytes, and very weak in periportal hepatocytes (Fig. 1B). When animals received repetitive injections (20 mg/kg × 4), the staining was strong in perivenular hepatocytes, moderate to strong in midzonal hepatocytes, and weak to moderate in periportal hepatocytes (Fig. 1C). When animals were injected singly (80 mg/kg) and killed 4 days after the injection, the staining was weak or moderate in perivenular hepatocytes and very weak in midzonal and periportal hepatocytes (Fig. 1D). If animals were injected singly with a large amount of PB (160 mg/kg) and killed 1 day after the injection, the staining was strong in perivenular hepatocytes, weak in midzonal hepatocytes, and very weak in periportal hepatocytes (Fig. 1E).

When the adjacent sections were incubated with normal serum instead of anti-CYP2B1/2 antibody, no staining was observed in hepatocytes and sinusoidal cells (Fig. 1F).

Under saturation conditions, immunostaining intensity resulting from CYP2B1/2 was almost proportional to the amount of the isoforms in sections when absorbance measured by image analysis was less than 0.3 [2, 3]. Thus, the intensity can be converted to molar content as follows:

$$\text{Antigen content (nmol/cm}^3\text{)} = (\text{SPIspec/ASIspec}) \cdot \text{BC/D}$$

where SIPspec = "specific immunostaining intensity in portions of sections" (=SIPab − SIPns), ASIspec = "average specific staining intensity in the whole section" (=ASIab − ASIns), BC = biochemically measured content (nmol/g liver), and D = specific gravity of the liver (1.07) [6, 13].

In the present study, ASIspec obtained by image analysis was 0.007 ± 0.0024 in control animals (mean ± SEM for 6 animals), 0.063 ± 0.0103 in animals injected singly, and 0.075 ± 0.0097 in those injected repetitively. CYP2B1/2 content in homogenate from control animals was 2.4 ± 0.18 (nmol/g liver; mean ± SEM for 6 animals), 19 ± 2.1 from animals injected singly, and 24 ± 2.3 from those injected repetitively. Therefore, the value "BC/(ASIspec · D)" was calculated to be almost constant (about 300) in control and the two PB-treated groups. Thus, if SIPspec is 0.100, CYP2B1/2 content (nmol/cm^3 cytoplasm), calculated by substituting these values into equation [1], is 30 nmol/cm^3 cytoplasm.

In control animals, CYP2B1/2 content was 6 ± 0.7 (nmol/cm^3 cytoplasm; mean ± SEM for 6 animals) in perivenular hepatocytes, 2 ± 0.4 in midzonal hepatocytes, and 2 ± 0.5 in periportal hepatocytes (Fig. 2). After the single injection, the content increased markedly in perivenular hepatocytes (62 ± 7.5; $P < 0.001$) and slightly in midzonal hepatocytes (15 ± 2.8; $P < 0.01$). However, the content was unchanged in periportal hepatocytes (3 ± 1.2). When animals received the repetitive injections, the content increased markedly in perivenular (60 ± 5.9; $P < 0.001$) and midzonal hepatocytes (43 ± 7.9; $P < 0.001$) and slightly in periportal hepatocytes (11 ± 1.8; $P < 0.01$).

Total CYP content was 57 ± 3.7 (nmol/cm^3 cytoplasm; mean ± SEM for 6 animals) in perivenular hepatocytes, 50 ± 3.8 in midzonal hepatocytes, and 39 ± 2.8 in periportal hepatocytes in control animals (Fig. 2). After the single injection, the content increased markedly in perivenular hepatocytes ($P < 0.001$) and slightly in midzonal hepatocytes ($P < 0.05$), whereas the content was unchanged in periportal hepatocytes. When animals received the repetitive injections, the content increased markedly in perivenular hepatocytes ($P < 0.005$), moderately in midzonal hepatocytes ($P < 0.01$), and slightly in periportal hepatocytes ($P < 0.05$).

The content of CYP isoforms other than CYP2B1/2, calculated by subtracting CYP2B1/2 content from total CYP content, was statistically unchanged after the single injection (Fig. 2). However, the content decreased in perivenular and midzonal hepatocytes ($P < 0.05$) after the repetitive injections, although the content was unchanged in periportal hepatocytes.

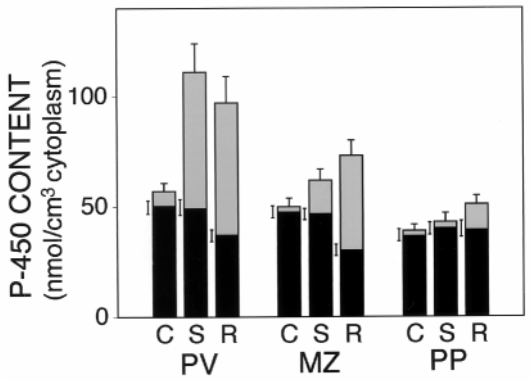

FIG. 2. Contents of total CYP, CYP2B1/2, and isoforms other than CYP2B1/2 in the hepatocyte cytoplasm of control and PB-treated rats. Measurements were done in the cytoplasm of perivenular (PV), midzonal (MZ), and periportal (PP) hepatocytes from control rats (C), animals injected singly with PB (80 mg/kg) (S), or animals injected repetitively with the drug at a dose of 20 mg/kg once a day for 4 days (R). Whole column, total CYP content; grey portion in each column, CYP2B1/2 content; and black portion in each column, isoforms other than CYP2B1/2. Values are the means of 6 animals. The vertical line on each column represents the SEM of total CYP content. The line at the side of each column shows the SEM of content of isoforms other than CYP2B1/2. The SEM of CYP2B1/2 isoforms in each experimental group is given in the text.

In control animals, the hybridization signal for CYP2B2 mRNA was scarce in perivenular hepatocytes and negligible in midzonal and periportal hepatocytes (Fig. 3A). The signal was negligible in sinusoidal cells. After the single injection of PB, the signal was strong in perivenular hepatocytes and weak or negligible in midzonal and periportal hepatocytes (Fig. 3B). When animals received the repetitive injections, the signal was strong in perivenular and midzonal hepatocytes and weak or moderate in periportal hepatocytes (Fig. 3C). When adjacent sections were incubated with sense probe or unlabeled antisense probe, the signal was negligible or very weak in hepatocytes and sinusoidal cells.

DISCUSSION

As shown in the present study, CYP2B1/2 isoforms increased markedly in perivenular hepatocytes, increased somewhat in midzonal hepatocytes, and remained unchanged in periportal hepatocytes after a single injection of PB (80 mg/kg × 1). However, although the isoforms increased markedly in perivenular hepatocytes, they also increased markedly in midzonal hepatocytes and somewhat in periportal hepatocytes when animals received repetitive injections of the drug (20 mg/kg × 4). Thus, although the animals of the two experimental groups received the same total dose of PB, the pattern of sublobular induction of

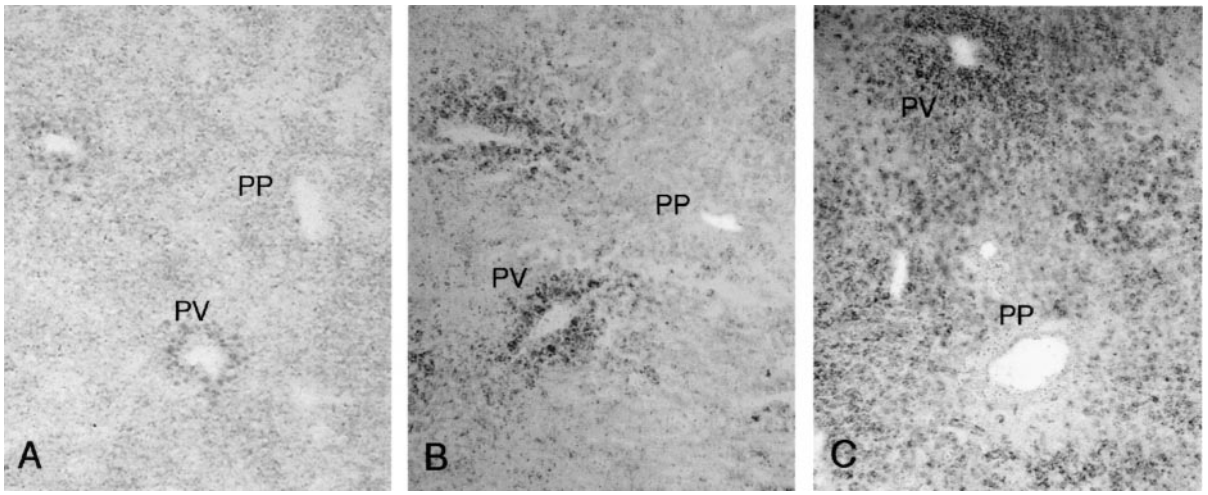

FIG. 3. ***In situ*** **hybridization for CYP2B2 mRNA in sections cut from the liver of a control rat (A), the liver of an animal injected singly with PB (B), and the liver of an animal injected repetitively with the drug (C). PP, periportal area; PV, perivenular area. Magnifications, ×40.**

CYP2B1/2 isoforms was different between the two groups. In addition, the isoforms were localized predominantly in perivenular hepatocytes 4 days after the single injection (80 mg/kg), showing that the difference in the pattern of induction was not due to the lapse of 4 days after the injection. Furthermore, the enzymes were localized predominantly in perivenular hepatocytes when induced by a single injection of 160 mg/kg of the drug. These findings indicate that the enlargement of the sublobular area in which induction of the isoforms occurred was caused primarily by the repetitive injections of PB.

There are two types of increase in amount of CYP isoforms after drug administration: (a) additive increase without reduction in other isoforms [2, 3, 14], and (b) substitutional increase with reduction in other forms [3, 8]. In the present results, CYP2B1/2 isoforms increased with a marked reduction in other isoforms in perivenular and midzonal hepatocytes (substitutional increase) after the repetitive PB injections, whereas the increase in periportal hepatocytes appeared to be additive. After a single injection, the isoforms increased in hepatocytes of the three zones without reduction of the total amount of other isoforms (additive increase). However, several CYPs such as CYP3A1/2 and 2C6 are also induced by PB treatment, although the amount of these isoforms has been shown to be smaller than that of CYP2B1/2 after PB treatment in rat liver [15]. It is unclear whether CYP2B1/2 increases without reduction in individual constitutive isoforms after the single injection, whereas a male-specific constitutive isoform, CYP2C11, has been shown to decrease in livers of rats after PB administration [16].

Hassett *et al.* [17] examined CYP2B1/2 expression in the liver of a rat after a single injection of 80 mg/kg of PB by *in situ* hybridization with a short (18-mer) oligonucleotide probe, and found that CYP2B1/2 (P450e and P450b) mRNA increases in the liver lobule except for the periportal region. That finding differs from the present results. In our preliminary experiments, however, the hybridization signal of CYP2B1/2 mRNA with the short probe was very weak, and the short probe formed many bands in northern blots. Thus, we could not use the short probe in the present study. The longer probe used in the present study formed a single band in northern blots [2].

The expression of *CYP2B1/2* genes has been presumed to be regulated at the transcriptional level through a *cis*-acting DNA element (PB-responsive element or PB-responsive enhancer module) [18–20], *trans*-acting factors [18], and intracellular PB receptors [21]. In addition, the expression of the genes has been shown to be regulated in certain cases at the post-transcriptional level [2, 22, 23]. In the present results, the pattern of increase in the CYP2B2 mRNA level appeared to be similar to that in the CYP2B1/2 protein level in hepatocytes of the three zones in animals injected both singly and repetitively with PB, suggesting that the expression of CYP2B1/2 isoforms is regulated at the transcriptional level. Recently, Honkakoski *et al.* [24], Kawamoto *et al.* [25], and Sueyoshi *et al.* [26] found that the constitutively active receptor–retinoid X receptor heterodimer activates a PB-responsive enhancer module in the distal enhancer element in *CYP2B* genes. The difference in the response of midzonal and periportal hepatocytes to PB stimulation between animals injected singly and repetitively can be explained by a difference in the expression of these receptors. In livers of normal animals, perivenular hepatocytes may already express large amounts of these receptors, but midzonal or periportal hepatocytes possibly express no receptors or very slight amounts. When animals receive repetitive injections of PB, the receptors might be expressed also in midzonal and periportal hepatocytes. This may result in induction of the isoforms in hepatocytes of all three zones after the repetitive PB injections.

Relative PB concentrations in the microenvironment possibly affect CYP2B1/2 induction in the different zones in the lobule. However, perivenular expression and perivenu-

lar induction of CYP2B1/2 is not attributed simply to this possibility because of the blood flow from the portal tract to the central (terminal hepatic) venule. In addition, Traber *et al.* [27] found that the expression of *CYP2B1/2* genes was induced 20- to 30-fold by PB treatment in only a fraction of hepatocytes that had been transplanted 6 months earlier into the spleen. Recently, however, Gupta *et al.* [28] examined expression of CYP2B1 in fractions of hepatocytes transplanted into the spleen, and found that the liver microenvironment exerted supremacy over the prior differentiation state of hepatocytes in directing position-specific *CYP2B1* gene expression. Thus, the effect of microenvironmental factors on zonal sublobular difference in CYP2B1/2 induction by PB cannot be ruled out. Oinonen *et al.* [29] found that the expression of CYP2B1/2 in the normally silent periportal area became high after hypophysectomy in rats, and replacement of growth hormone re-established perivenularly restricted expression. Thus, growth hormone may regulate the sublobular expression of *CYP2B1/2* genes in the livers of rats.

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