

Activation of Cytochrome P450 Gene Expression in the Rat Brain by Phenobarbital-Like Inducers¹

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Accepted for publication May 11, 2000 This paper is available online at <http://www.jpet.org>

ABSTRACT

Oxidative biotransformation, coupled with genetic variability in enzyme expression, has been the focus of hypotheses interrelating environmental and genetic factors in the etiology of central nervous system disease processes. Chemical modulation of cerebral cytochrome P450 (P450) monooxygenase expression character may be an important determinant of in situ metabolism, neuroendocrine homeostasis, and/or central nervous system toxicity resulting from exposure to neuroactive drugs and xenobiotic substances. To examine the capacity of the rat brain to undergo phenobarbital (PB)-mediated induction, we developed reverse transcription-polymerase chain reaction methods and evaluated the effects of several PB-like inducers on P450 and microsomal epoxide hydrolase gene expression. Animals treated i.p. with four daily doses of PB demonstrated markedly induced levels of CYP2B1, CYP2B2, and CYP3A1

mRNA in the striatum and cerebellum. In contrast, 1 or 2 days of PB treatment resulted in unchanged or even slightly decreased levels of CYP2B1 and CYP2B2 in the brain, although the latter treatments produced marked induction of the corresponding genes in the liver. Only slight increases in epoxide hydrolase RNA levels resulted in brains of PB-treated animals. Substantial activation of cerebral CYP2B1, CYP2B2, and CYP3A1 mRNA levels also resulted when animals were treated with the neuroactive drugs diphenylhydantoin and amitriptyline, and with the potential PB-like xenobiotic inducers *trans*-stilbene oxide and diallyl sulfide, whereas dichlorodiphenyltrichloroethane was less efficacious. Although the time course of the induction response is delayed in brain relative to that required for the liver, these results clearly establish that brain P450s are markedly PB inducible.

Characterization of the modulatory factors affecting the vulnerability of the central nervous system (CNS) to chemical insult is of critical neurotoxicological importance. Metabolism of toxicants directly in brain tissues may be largely mediated by enzymes in the cytochrome P450 (P450; EC 1.14.14.1) superfamily (Warner et al., 1988; Ravindranath, 1998). In most cases, P450-dependent metabolism leads to detoxification, conjugation, and excretion of chemical agents. However, electrophilic intermediates are sometimes formed that bind proteins, nucleic acid, or lipids (Guengerich and Leibler, 1985; Strobel et al., 1997). Because neurons have limited regenerative capacity, oxidative injury of these cells may result in irreparable cellular damage.

Several lines of evidence have indicated a possible associ-

ation between neurodegenerative diseases, including Alzheimer's and Parkinson's disease, and exposure to drugs and environmental or occupational substances (Ludolph, 1995; Strobel et al., 1997; Manzo and Costa, 1998). P450 enzymes, and genetic variability in enzyme expression, have been the focus of hypotheses interrelating environmental and genetic factors in the etiology of CNS disease processes (Shahi et al., 1992; Riedl et al., 1998, 1999). Modulation of biotransformation enzyme expression through the process of gene induction is a potentially important factor impacting drug or chemical detoxication (Okey et al., 1986). Induction of P450s coupled with enhanced in situ metabolism in the brain may therefore contribute to local toxicity and neuronal degeneration (Strobel et al., 1997).

In a previous study we used reverse transcription-polymerase chain reaction (RT-PCR) approaches to detect and estimate levels of several P450 and microsomal epoxide hydrolase (EH) mRNAs in different regions of the rat brain (Schilter and Omiecinski, 1993). We demonstrated the inducibility of brain CYP1A1 and CYP1A2 mRNA in β -naphthoflavone-treated rats after single-dose administration of the inducer. We also deter-

Received for publication January 7, 2000.

¹ This work was supported by Grant GM 32281 from the National Institute of General Medical Sciences and by National Institute of Environmental Health Sciences Center Grant ES07733. B.S. was a recipient of a Swiss National Research Foundation fellowship. C.J.O. is a Burroughs Wellcome Fund Toxicology Scholar.

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ABBREVIATIONS: CNS, central nervous system; P450, cytochrome P450; RT-PCR, reverse transcription-polymerase chain reaction; EH, microsomal epoxide hydrolase; PB, phenobarbital; QC, quantitative competitive; DPH, diphenylhydantoin; AMI, amitriptyline; DDT, dichlorodiphenyltrichloroethane; TSO, *trans*-stilbene oxide; DAS, diallyl sulfide; rcRNA, recombinant competitive RNA; FP, forward primer; RP, reverse primer.

mined that a single dose of phenobarbital (PB), administered i.p., was sufficient to increase levels of CYP2B1 in the medulla oblongata, midbrain, and cortex. However, confounding data were generated indicating that CYP2B1 mRNA levels in the cerebellum, striatum, and hypothalamus were decreased with this PB treatment, as were CYP2B2 mRNA levels in all areas of the brain. Overall, the analysis of P450 induction in various brain regions after an acute exposure of PB revealed a heterogeneous pattern (Schilter and Omiecinski, 1993).

In this investigation we expanded these analyses by investigating the effects of multiple PB-dosing schemes on CYP2B1, CYP2B2, CYP3A1, and EH mRNA levels in the rat brain and liver. RT-PCR-based assays were used to detect and quantify the low mRNA levels in rat cerebellum and striatum, regions that were refractive to PB induction after single exposures to the inducer (Schilter and Omiecinski, 1993). In addition to semiquantitative analyses, we developed a novel quantitative competitive RT-PCR (QC RT-PCR) assay with a multispecific and polyadenylated internal standard RNA molecule that enabled accurate assessment of P450 and EH mRNA levels. In addition, we investigated the effects of other structurally diverse CYP2B inducers, including diphenylhydantoin (DPH), amitriptyline (AMI), dichlorodiphenyltrichloroethane (DDT), *trans*-stilbene oxide (TSO), and diallyl sulfide (DAS) on brain and liver expression patterns. Our results indicate that P450 gene expression in the brain is markedly activated by exposures to several PB-like inducers, although the kinetics of the induction process differs considerably from that occurring in the liver.

Materials and Methods

Chemicals. PB sodium, DPH, AMI, DDT, TSO, and DAS were obtained from Sigma Chemical Co. (St. Louis, MO). For animal injections PB, DPH, and AMI were dissolved in saline solution, whereas DDT, TSO, and DAS were dissolved in corn oil.

Animals and Operative Procedures. Adult male Sprague-Dawley rats (280–320 g) were obtained from Simonsen (Gilroy, CA). Animals were maintained on a 12-h light/dark cycle with food and water available ad libitum. Intraperitoneal injections of PB were administered once daily (80 mg/kg) for 1, 2, or 4 days. DPH (100 mg/kg), AMI (10 mg/kg), DDT (200 mg/kg), TSO (300 mg/kg), and DAS (200 mg/kg) were injected i.p. once daily for 4 days. Control animals received either no injections or injections with the respective vehicle. Animals were sacrificed 18 h after the final injection by decapitation under ether anesthesia. Brains were rapidly removed and washed in ice-cold saline. The cerebellum and the striatum were dissected according to Glowinski and Iversen (1966). Liver and brain tissues were frozen immediately in liquid nitrogen and stored at -80°C . All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by our Institutional Animal Care Committee.

Isolation of Total RNA. Total RNA was isolated from frozen rat liver and brain tissues with the TRIzol Reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol and as described previously (Chomczynski and Sacchi, 1987). The RNA samples derived were dissolved in nuclease-free water and quantified with UV-spectrophotometry at 260 nm.

Slot Blot and Northern Blot Analyses. Aliquots (5 μg) of liver RNA were transferred to GeneScreen Plus (DuPont, Wilmington, DE) membranes and probed with ^{32}P -labeled oligonucleotides that specifically hybridize to CYP2B1, CYP2B2, CYP3A1 mRNA, and 18S RNA (Omiecinski et al., 1990; Schilter and Omiecinski, 1993). North-

ern blot analyses also were conducted with 1.5% agarose-2.2 M formaldehyde gels to verify RNA integrity, concentration determinations, and specificity of hybridization probes (data not shown).

Semiquantitative RT-PCR Analyses. Semiquantitative RT-PCR analysis was performed as described previously (Schilter and Omiecinski, 1993). Briefly, 5 μg of total RNA was reverse transcribed with Superscript RNaseH-reverse transcriptase (Life Technologies) according to the manufacturer's protocol with oligo(dT)₁₂₋₁₈ primer. A dilution series of cDNA was amplified in PCR reactions consisting of 1 \times *Taq* polymerase buffer with 1.5 mM MgCl₂ (Promega, Madison, WI), 200 μM each dNTP, 10 pmol of each primer pair, and 1 U of *Taq* DNA polymerase (Promega). The samples were cycled 30 times through a 40-s denaturation at 93 $^{\circ}\text{C}$, 40-s annealing at 54 $^{\circ}\text{C}$, and 40-s extension at 72 $^{\circ}\text{C}$. To assess potential DNA contamination, RT minus controls were included for all samples. A portion of each PCR was electrophoresed in ethidium bromide-stained 2% agarose gels and then Southern blotted and hybridized with ^{32}P -labeled oligonucleotides. Each experiment was repeated with two animals, generating new cDNAs at least two times and repeating each PCR reaction at least twice. Representative results are presented.

Construction of the QC RT-PCR Standard. A recombinant plasmid (pRATP450) was developed to facilitate quantitative measurements of P450 and EH mRNAs. The RNA transcribed from pRATP450 was used as an internal standard in QC RT-PCR assays. The recombinant competitive RNA (rcRNA) molecule is 922 bases in length and composed of 16 PCR primer-binding sites, 8 hybridization probe-binding sites, a 297-base spacer sequence, and a 50-base poly(A) tail. The primers selected for use in the synthetic template standard are indicated in Table 1 and were designed to flank intron sequences and were optimized to specifically amplify both native and standard cDNA. The amplified products resulting from the rcRNA molecules are approximately 35% longer than those derived from native mRNA.

The primer-binding portion of pRATP450 was constructed from eight overlapping oligonucleotides (Genosys, The Woodlands, TX) each 70 to 85 bases in length. Oligonucleotides were purified from polyacrylamide gels with UV shadowing. Two sets of four overlapping oligonucleotides were amplified by PCR according to Tarnuzzer et al. (1996) with modifications. Five hundred nanograms of oligonucleotides 1 to 4 was added to a 100 μl of PCR containing 200 μM each dNTP, 2.6 U of Expand High Fidelity DNA polymerase, and 1 \times Expand High Fidelity PCR buffer (Boehringer Mannheim, Indianapolis, IN). This reaction was cycled seven times in an MJ DNA Engine (MJ Research, Watertown, MA) thermal cycler with a heated bonnet by incubating at a calculated temperature of 94 $^{\circ}\text{C}$ for 7 min followed by a 58 $^{\circ}\text{C}$ annealing for 20 s and a 72 $^{\circ}\text{C}$ extension for 30 s. Two microliters of the first reaction was added to another 100 μl of PCR containing 0.3 μM each of forward primer (FP) 1 and oligonucleotide 4 and amplified an additional 20 cycles. This procedure was repeated for oligonucleotides 5 to 8 adding oligonucleotide 5 and reverse primer (RP) 8 in the 20-cycle amplification. Finally, 2 μl of each reaction was added to another 100 μl of PCR reaction with 0.3 μM FP1 and RP8. The latter reactions were heated to 94 $^{\circ}\text{C}$ for 2 min followed by 35 cycles of a 30-s denaturation at 94 $^{\circ}\text{C}$, 40-s annealing at 55 $^{\circ}\text{C}$, and a 50-s extension at 72 $^{\circ}\text{C}$. After the final cycle, reactions were incubated for 5 min at 72 $^{\circ}\text{C}$.

The final amplification resulted in a 514-base cassette that was cloned into pBluescript II KS+ (Stratagene, La Jolla, CA) along with 50 bases of poly(A). A 297-base fragment of the *E. coli* chloramphenicol acetyl transferase gene was used as a spacer by cloning into the vector between the primer sites and the poly(A) sequence making the rcRNA standard similar in length to native mRNA molecules. Finally, the integrity of pRATP450 was confirmed by four-color fluorescent dideoxy terminator cycle sequencing analysis with an ABI Prism 377 DNA sequencer (PE/Applied Biosystems, Inc., Foster City,

TABLE 1

P450 and EH oligonucleotides contained in the synthesized pRATP450 standard and native cDNA

The QC standard contains primer sets for seven distinct rat P450s and for EH. The QC standard is used to generate a synthetic RNA molecule that possesses a poly(A) tract. A known amount of synthetic RNA is then added to samples of native RNA and the mixture is reverse transcribed with (oligo)dT primers. The QC standard PCR products from the resulting cDNAs are approximately 35% longer than the product sizes that are derived from native cDNA amplimers to facilitate their separation by gel electrophoresis. The sizes of the respective PCR products also were designed to yield different sizes for each respective gene.

cDNA	Oligo Sense	Oligo Name	Oligonucleotide Sequence 5' to 3'	STD PCR BP	Native PCR BP	Ratio
CYP1A1	FP	CYP1A1FP	CCATGACCAGGAACATATGGG	460	341	1.35
	RP	CYP1A1RP	TCTGGTGAGCATCCAGGACA			
	HP	CYP1A1HP	AGCCTGGAGATGCTGAGGAC			
CYP1A2	FP	CYP1A2FP	CTGCAGAAAACAGTCCAGGA	139	18	1.18
	RP	CYP1A2RP	GAGGGATGAGACCACCGTTG			
	HP	CYP1A2HP	CTCACTGTGCTTGAACAGGG			
CYP2B1	FP	CYP2B1FP	GCTCAAGTACCCCCATGTCTG	144	109	1.32
	RP	CYP2B1RP	ATCAGTGTATGGCATTCTACTGCGG			
	HP	CYP2B1HP	GGTTGGTAGCCGGTGTGA			
CYP2B2	FP	CYP2B2FP	CTTTGCTGGCACTGAGACCG	207	162	1.27
	RP	CYP2B2RP	ATCAGTGTATGGCATTCTGGTACGA			
	HP	CYP2B2HP	GGATGGTGGCCTGTGAGA			
CYP2E1	FP	CYP2E1FP	GGATGTGACTGACTGTCTCC	447	321	1.30
	RP	CYP2E1RP	TGGGGTAGGTTGGAAGGGAC			
	HP	CYP2E1HP	CTGCAAAGAACAGGTCGGCC			
CYP3A1	FP	CYP3A1FP	CCGCCTGGATTCTGTGCAGA	376	263	1.43
	RP	CYP3A1RP	TGGGAGGTGCCTTATTGGGC			
	HP	CYP3A1HP	CGGATAGGGCTGTATGAGATTC			
CYP4A1	FP	CYP4A1FP	AGCAGTCCCATCACCTCCC	502	391	1.28
	RP	CYP4A1RP	TCTTGACCTGCCAGCTGTTC			
	HP	CYP4A1HP	GGTTAGCATTGCGCCGGTGTCT			
EH	FP	MEHFP	CACATCCAAGCCACCAAGCC	252	135	1.86
	RP	MEHRP	CAGGCCTCCATCTCCAG			
	HP	MEHHP	GGTACTCTGACTTGGTCCAG			

Oligo, oligonucleotide; STD, internal standard; BP, base pairs; Ratio, standard QC PCR size/native PCR size; HP, hybridization probe.

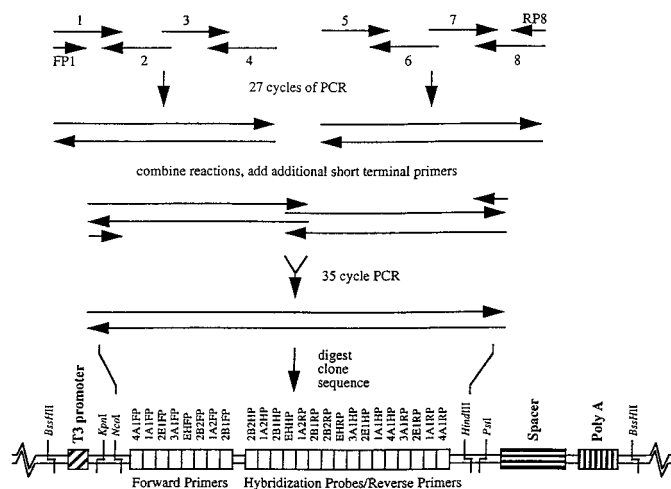


Fig. 1. Construction of pRATP450. Eight long, overlapping oligonucleotides were used to construct the primer-binding portion of pRATP450. Oligonucleotides 1 to 4 and 5 to 8 were amplified with two-step PCRs as described under *Materials and Methods*. A portion of these two reactions was combined and amplified with FP1 and RP8 to yield a 514-base cassette that was cloned into pBluescript II KS+. HP, hybridization probe.

CA). One point mutation was corrected with site-directed mutagenesis. The construction strategy and structural features of the synthetic standard are depicted in Fig. 1.

Synthesis of rcRNA. Synthetic rcRNA was generated in an *in vitro* transcription assay with a MAXIScript kit (Ambion Inc., Austin, TX) according to the enclosed protocol. Briefly, 5 μ g of *Bss*HIII (New England Biolabs, Inc., Beverly, MA) digested pRATP450 was incubated with 50 U of T3 RNA polymerase, 1 \times transcription buffer, and 0.5 mM each NTP at 37°C for 1 h. [α - 32 P]UTP (1 μ Ci; DuPont NEN Research Products, Boston, MA) was included in the reaction for quantification of the rcRNA. The DNA template was digested by treating with RNase-free DNase I (Ambion Inc.), and full-length

rcRNA was isolated with the PolyAtract mRNA Purification system IV (Promega). Purified rcRNA was quantified by liquid scintillation counting. Yeast tRNA was added to the purified rcRNA as a carrier. An aliquot of the rcRNA was electrophoresed in a polyacrylamide gel to confirm its length and integrity.

QC RT-PCR Analysis. QC RT-PCR analyses with an internal RNA standard were performed as described previously (Andersen et al., 1998). A dilution series of rcRNA (1.58×10^4 – 5.00×10^8 copies) was added to 100-ng (liver) or 1.00- μ g (brain) aliquots of total RNA. Mixtures of RNA were treated with DNase I and cDNA was synthesized with SuperScript II RNaseH-Reverse Transcriptase (Life Technologies) according to the enclosed protocol with oligo(dT)₁₂₋₁₈ primer. Control reactions without enzyme were performed for each RNA sample. A small portion of each RT reaction (5%) was amplified in 10 μ l of PCRs containing 1 \times PCR buffer (Life Technologies), 1.5 mM MgCl₂, 200 μ M each dNTP, 0.5 μ M each of a primer pair listed in Table 1, and 0.2 U of recombinant *Taq* DNA Polymerase (Life Technologies). All batches of PCR included template-minus controls. Competitive PCRs were performed by heating samples to a calculated temperature of 94°C for 2 min followed by 40 cycles of a 20-s denaturation at 94°C, 20-s annealing at 58°C, and a 30-s extension at 72°C. After the final cycle, reactions were incubated for 5 min at 72°C. A portion of each PCR was electrophoresed in ethidium bromide-stained 2% agarose gels. A Gel Doc 1000 UV Fluorescent Gel Documentation system (Bio-Rad, Hercules, CA) was used to visualize DNA bands and generate digital images of gels. Quantitative band analysis was performed with Molecular Analyst version 2.1.1 (Bio-Rad). The initial number of target mRNAs was calculated as described previously (Andersen et al., 1998). Reactions with each pair of PCR primers produced bands of predicted size for native and standard RNA (Table 1). The specificity of the products generated was verified with Southern blotting with 32 P-labeled hybridization probes (Table 1) that anneal to expected internal unique sequences present in native and standard PCR products (data not shown). The specificity of several of the RT-PCR primer sets and corresponding internal hybridization probes used in the current assays also have been characterized previously (Omiecinski et al., 1990a,b).

Results

PB-Inductive Effects in the Brain. We used two different RT-PCR-based assays to detect the estimated low levels of P450 mRNA in rat brain and to investigate the effects of PB on the cerebral expression of CYP2B1, CYP2B2, CYP3A1, and EH. For the semiquantitative approach, we performed preliminary experiments to determine the proper cDNA dilution to ensure linear amplification, as described previously (Schilter and Omiecinski, 1993). Semiquantitative analyses of P450 and EH mRNA levels in PB-treated rats over 4 days are presented in Fig. 2. One or 2 days of PB treatment resulted in decreased levels of CYP2B2 mRNA in the striatum and cerebellum, whereas CYP2B1 was decreased only in the striatum. However, 4 days of PB treatment resulted in large increases in levels of CYP2B1, CYP2B2, and CYP3A1 mRNA in both the cerebellum and striatum. EH mRNA levels were only slightly elevated in the brains of PB-treated animals in the same treatment groups.

We confirmed and extended the results of the semiquantitative RT-PCR analyses of PB induction with a QC RT-PCR assay (under *Materials and Methods*). Results with this method are not affected by amplification beyond the linear phase and the use of radioisotopes is not required. A version of this assay specifically designed for human P450 and EH sequences was used successfully to determine gene expression levels of an array of biotransformation enzymes in human liver and human peripheral lymphocytes (Andersen et al., 1998; Krovat et al., 2000). The quantitative data obtained for brain and liver samples are presented in Fig. 3. Liver CYP2B1 and CYP2B2 levels were elevated 340- and 55-fold, respectively, after 4 days of PB treatment. Levels of CYP2B1 and CYP2B2 mRNA also were markedly increased in both the cerebellum and striatum. In the induced state, CYP2B1

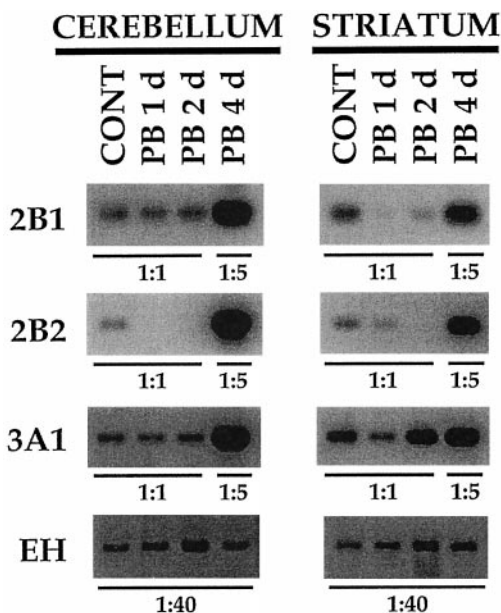


Fig. 2. Effects of repeated PB treatment on cerebral P450s and EH. Semiquantitative RT-PCR was performed on RNA from the cerebellum and striatum of control rats (CONT) or from rats injected with PB (80 mg/kg) once a day for 1, 2, or 4 days. cDNAs were diluted as indicated before PCR amplification to establish linear phase amplification conditions (under *Materials and Methods*; Schilter and Omiecinski, 1993). Southern blotted PCR products were probed with ^{32}P -labeled oligonucleotides specific to the respective mRNAs.

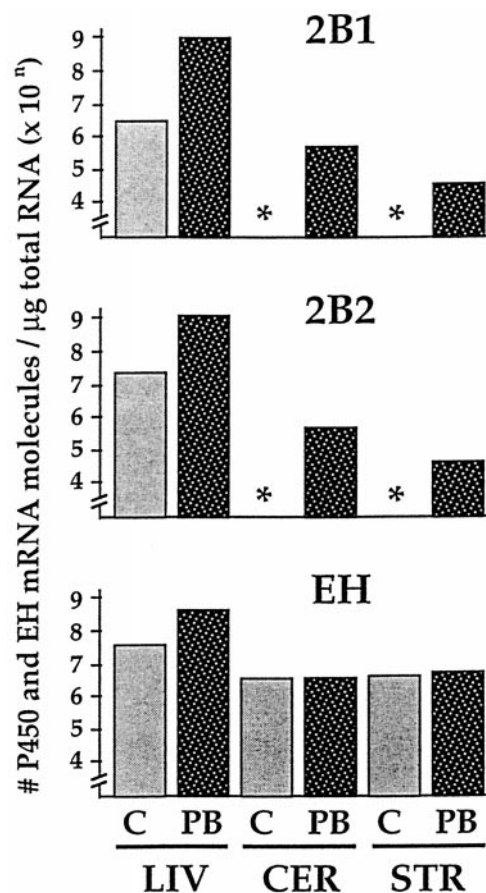


Fig. 3. Effects of subchronic PB treatment on brain and liver P450s. QC RT-PCR was performed on RNA from liver (LIV), cerebellum (CER), and striatum (STR) of rats injected with saline (C) or PB (80 mg/kg) once a day for 4 days. Values are the average of two measurements; standard deviations ranged from 3 to 30%. *, $<1.58 \times 10^4$ mRNA molecules/ μg . See *Materials and Methods* and Andersen et al. (1998) for details of the QC RT-PCR assay procedure.

mRNA levels were nearly equivalent to constitutive CYP2B1 mRNA levels in the liver. Basal P450 mRNA in the brain was below the limit of detection for this assay (1.58×10^4 mRNA molecules/ μg). In contrast, EH mRNA was detected at high levels in all tissues examined. Brain EH mRNA levels did not change substantially with 4 days of PB treatment, whereas liver EH levels increased approximately 12-fold.

Effects of Other Inducers on Brain mRNA Levels. Several other potentially PB-like inducing compounds also evaluated for their effects on brain and liver expression. With a protocol similar to PB, rats were injected once a day for 4 days with DPH (100 mg/kg) or AMI (10 mg/kg). The cerebral expression of CYP2B1, CYP2B2, CYP3A1, and EH was then compared with those of controls and rats receiving 4 days of PB treatment. The data in Fig. 4A demonstrate that CYP2B1, CYP2B2, and CYP3A1 mRNA levels increased in animals receiving DPH and AMI, relative to the control animals. DPH induced cerebral P450s almost as efficiently as PB, whereas AMI at this dose was less effective. The effects of 4-day treatments with other PB-like inducers, DDT, TSO, and DAS, are presented in Fig. 4B. Elevated P450 levels resulted in brains of TSO- and DAS-treated animals, whereas DDT increased levels of CYP2B1 and CYP2B2 mRNA only in the cerebellum. Cerebral EH mRNA levels

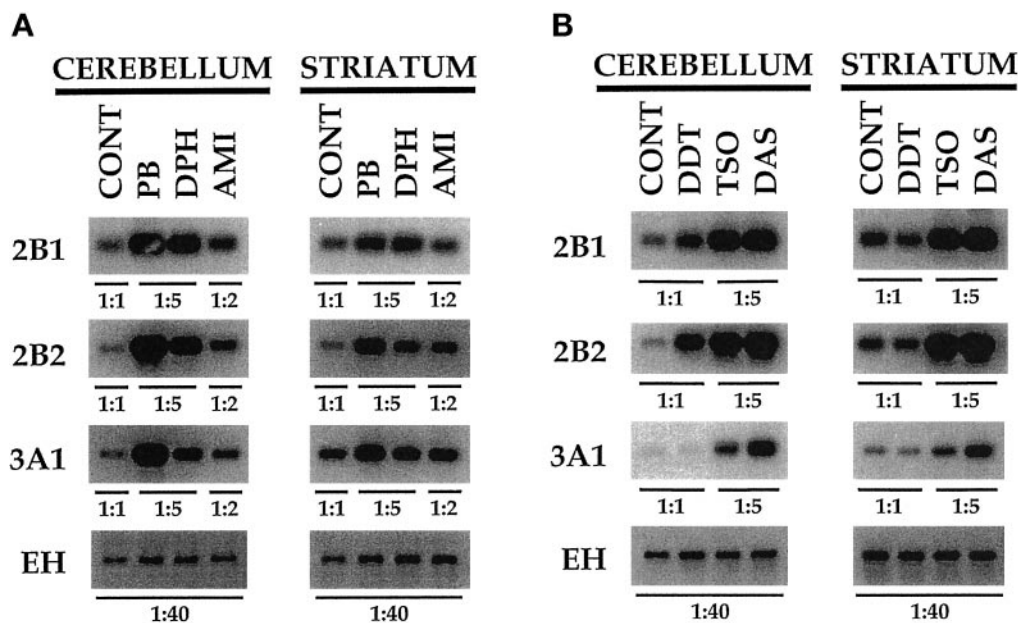


Fig. 4. Comparison of the effects of potential PB-like inducers on cerebral P450s and EH. Semiquantitative RT-PCR was performed on RNA from the cerebellum and striatum of rats injected once a day for 4 days with saline (CONT), PB (80 mg/kg), DPH (100 mg/kg), or AMI (10 mg/kg) (A) or once a day for 4 days with corn oil (CONT), DDT (200 mg/kg), TSO (300 mg/kg), or DAS (200 mg/kg) (B). cDNAs were diluted as indicated before PCR amplification to establish linear phase amplification conditions (under *Materials and Methods*; Schilter and Omiecinski, 1993). Southern blotted PCR products were probed with ³²P-labeled oligonucleotides specific to the respective mRNA.

were not substantially altered with the treatments of any of these test agents, and thus served as an effective internal control throughout the studies.

Hepatic Effects. Figure 5 presents the results of slot blot analyses for liver RNA samples from rats treated with various inducers probed with oligonucleotides specific to CYP2B1, CYP2B2, CYP3A1, or 18S RNA (Schilter and Omiecinski, 1993). As expected, CYP2B1, CYP2B2, and CYP3A1 were elevated after the first injection of PB and remained at high levels through 4 days of treatment. Among the other treatments, DDT, TSO, and DAS appeared simi-

larly effective as PB with respect to induction capacity for these hepatic P450s, whereas DPH was somewhat less potent. As for the brain, AMI was not a very effective inducer of hepatic P450s.

Discussion

Certain transcriptional activators, such as the PB class of inducing compounds, are well recognized for their gene-inductive effects in the liver. However, induction responses to PB-like agents in other organs are typically absent (Omiecinski, 1986; Waxman and Azaroff, 1992). Although the levels of most biotransformation enzymes are very low in the CNS, relative to the liver (Kapitulnik et al., 1987; Köhler et al., 1988), localized metabolism of neuroactive drugs or endogenous hormones may have a substantive impact on functional responses and/or neurotoxic events occurring subsequent to exposure challenge (Glowinski and Iversen, 1966; Gram et al., 1986; Ludolph, 1995). To accurately examine the expression character of these genes in the CNS, it is necessary to adopt highly sensitive detection schemes. In this study, we used a series of RT-PCR-based methods to assess P450 and EH mRNA levels in specific regions of the rat brain as a function of pretreatment with several agents of clinical and toxicological relevance. Our initial strategy involved a semi-quantitative RT-PCR approach (Omiecinski et al., 1990; Schilter and Omiecinski, 1993). Subsequently, we designed a novel QC RT-PCR procedure to more exactly measure the relative changes occurring in gene transcript levels after inducer exposures. The latter methodology was modeled after a similar QC RT-PCR approach used successfully to quantify expression of a battery of human P450s (Andersen et al., 1998). Thus, in this investigation, a rat standard was similarly constructed and characterized (Andersen et al., 1988) and the QC RT-PCR method was applied to examine the expression character of three prototypical PB-inducible P450s and EH in discrete regions of the rat brain.

In a previous study, we evaluated the responsiveness of several P450 genes in various anatomical regions of the rat brain (Schilter and Omiecinski, 1993). Although 1 day of PB

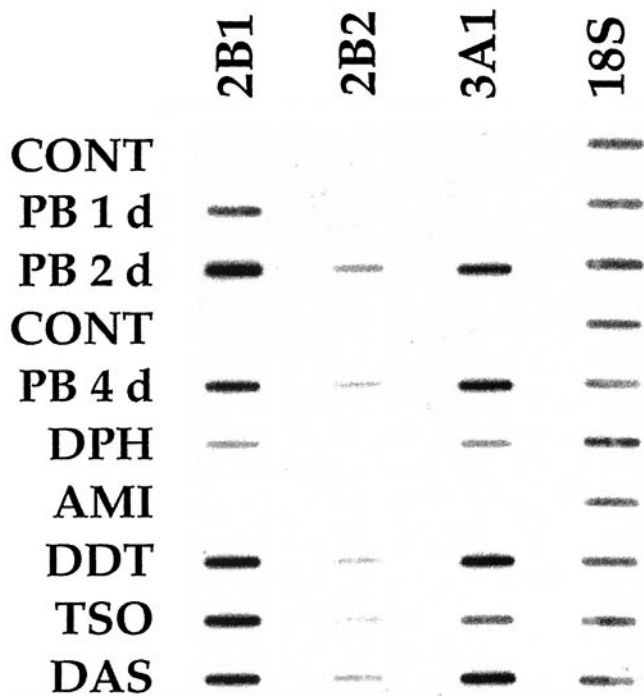


Fig. 5. Induction of hepatic P450s by PB-like inducing chemicals. Slot-blot hybridization analyses were performed on total liver RNA from rats treated with vehicle (CONT), PB (1, 2, or 4 days), and other inducers (4 days). See *Materials and Methods* for dosing regimens and hybridization details.

treatment resulted in elevated CYP2B1, CYP2B2, and CYP3A1 mRNA in the liver, decreased levels were found in the cerebellum and striatum (Schilter and Omiecinski, 1993). These studies were extended in this investigation to more thoroughly evaluate the time course kinetics of the induction process, in particular with respect to the potential impact of multiple daily dosing of PB-like inducers. Similar to our previous results, single treatments with PB did not result in a detectable induction response in the cerebellum or striatum (Fig. 2). However, monitoring animals treated with multiple doses of PB revealed tissue-specific and time-dependent enhancement in expression. CYP3A1 was the first gene product in the brain to demonstrate increased mRNA expression after PB treatment, with elevated mRNA levels detected in the striatum after 2 days of inducer administration (Fig. 2). After four consecutive days of treatment, marked elevations were evident in the levels of mRNA CYP2B1, CYP2B2, and CYP3A1 in both the cerebellum and striatum. Although drastically elevated relative to untreated controls, the measured levels of P450 mRNA in the induced brain were still much lower than that existing constitutively in the liver (Fig. 3). In contrast, cerebral EH mRNA levels were not altered substantively in the brain at any point in the treatment period.

We also demonstrated that induction of P450s in the brain is not limited to PB. Several other chemicals produced dramatic increases in brain mRNA levels, including compounds that were not particularly effective inducers of P450s in the liver. For example, DPH marginally induced P450s in the liver (Fig. 5), whereas in the brain it was nearly as effective as PB (Fig. 4A). In contrast, the halogenated pesticide DDT caused a large increase in P450 mRNA in the liver, whereas in the brain DDT was less potent (Figs. 5 and 4B, respectively). The basis for this apparent discrepancy of responses between the brain and the liver is not yet understood, but may involve distributional or dispositional kinetic differences.

The results presented are important in a number of respects. First, we demonstrated that subacute exposure to PB markedly induces the expression of CYP2B1, CYP2B2, and CYP3A1 mRNA in the rat brain. With the quantitative measures of RT-PCR, it is clear that although basal P450 mRNA levels are much lower in the brain, the capacity of induction is comparable. These findings contradict an established perception that extrahepatic tissues, with the exception of intestinal enterocytes (Traber et al., 1988), are not responsive to PB. Moreover, the results of these experiments suggest that the timing and, perhaps dispositional kinetics, of the PB response in the brain do not parallel the liver. Although liver P450s are induced relatively soon after a single PB treatment, brain P450s are unaltered or even decreased after 2 days of treatment, but exhibit substantial induction after 4 days of treatment. The mechanism for this delayed cerebral response may be the result of pharmacokinetic factors, potentially requiring longer treatment intervals to attain effective drug levels across the blood-brain barrier. PB also has been reported to interact with the λ -aminobutyric acid_A receptor, causing acute neurological effects that lead to a tolerance time frame (Saunders and Ho, 1990; Yu and Ho, 1990). Thus, there may be a brief interference between the receptor-mediated neuroactive effects of PB and its CYP2B induction pathway in the brain that is overcome with longer PB treatment regimens.

Recently, other investigators have reported a 3- to 4-fold increase in pentoxyresorufin-*O*-dealkylase activity in rat brain after 5 days of i.p. treatment with PB (Parmar et al., 1998). This activity marker is generally associated with CYP2B and CYP3A catalysis (Sidhu et al., 1993). Another recent study further demonstrated that testosterone metabolism in the rat brain is enhanced by DPH-inducible P450 isoforms (Rosenbrock et al., 2000). Although no time course kinetics or regioselective effects were assessed in the latter investigations, the results corroborate the findings reported herein and further indicate that the enhanced levels of P450 mRNA expression measured in the induced rat brains reflect functional activities in this organ. A report examining the PB-inducible human P450 counterpart CYP2B6 indicated that this latter protein was detectable at low levels, constitutively, in human brain (Gervot et al., 1999). We and others have demonstrated previously that certain P450s and EH are expressed in the human brain (Farin and Omiecinski, 1993; Ghersi-Egea et al., 1993). Thus, the results reported herein with the rat model are likely relevant to human exposures to PB-like agents as well.

In summary, in this investigation we demonstrated that rat brain P450s exhibit a unique response to PB challenge. In conjunction with results from our previous study (Schilter and Omiecinski, 1993), we have established that regional and gene-specific responses occur within the rat brain to several chemicals possessing varied structure, including several neuroactive drugs. The structural diversity of PB-like inducers is a striking feature of this induction pathway (Lubet et al., 1992; Waxman and Azaroff, 1992). In contrast to the liver, we demonstrate that a substantially longer duration of PB treatment is required to induce CYP2B1 and CYP2B2 mRNA in the brain. Due to the high concordance between elevated CYP2B mRNA and increased functional activity (Waxman and Azaroff, 1992; Sidhu et al., 1994; Parmar et al., 1998), it is reasonable to conclude that the marked increases in levels of P450 gene expression stimulated by the PB inducers likely result in functional alterations on CNS steroidogenesis and/or other local metabolic events occurring in the mammalian brain. In this regard, a recent report indicated that PB treatment may elevate neuronal nitric oxide synthase expression in the rat cerebellum (Thompson et al., 1997). Investigators studying the effects of neurotoxins or neuroactive pharmaceuticals should consider the delayed P450 response in the brain and bear in mind that the time course of the exposure may modulate the tolerance, disposition, and/or toxicity of xenobiotics.

Acknowledgments

We thank L. Aicher for technical assistance and Drs. J. S. Sidhu, F. M. Farin, and B. Krovat for assistance and helpful discussions.

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