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DISTINCT INDUCTION PROFILES OF THREE PHENOBARBITAL-RESPONSIVE MOUSE LIVER CYTOCHROME P450 ISOZYMES

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Abstract—The dose- and time-responses of three liver cytochrome P450 (P450) isozymes to 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) and phenobarbital (PB) were studied in DBA/2 mice at activity, protein and mRNA levels. We found that the maximal induction ranged from about 3-fold (P4502a-4/5) and 5-fold (P4502c-x) to more than 50-fold (P4502b-10). Only P4502a-4/5 and associated mRNA displayed a biphasic time-response after TCPOBOP induction: a transient increase occurring 3–8 hr after administration with a subsequent decline at 24 hr before the maximal induction at 72 hr. The changes in P450 isozyme content reflected those in mRNA levels suggesting that the induction by TCPOBOP and PB is controlled largely at pretranslational stages. The isozyme P4502c-x and associated immunoinhibited benzphetamine *N*-demethylase and testosterone 16β-hydroxylase activities were induced half-maximally by 6–30 times smaller doses of TCPOBOP and by three to four times smaller doses of PB than isozymes P4502a-4/5, P4502b-10 or related activities. Furthermore, larger doses of TCPOBOP decreased the expression of P4502c-x to sub maximal levels. Our data show that the three isozymes, although all inducible by TCPOBOP and PB, have distinct dose dependencies and different time-responses to induction. This indicates that the induction by TCPOBOP and PB of P450s belonging even to the same subfamily may proceed by different mechanisms.

Phenobarbital (PB‖) induces several hepatic cytochrome P450 (P450) isozymes belonging to gene subfamilies 2B, 2C, 2A and 3A¶ [1, 2]. In most cases, the induction is due to a stimulated transcription of the gene [3–5]. Despite considerable effort, the factors responsible for PB induction have not been identified and the exact mechanism remains unknown. Studies with rat hepatocyte cultures indicate that PB may induce 2B and 3A mRNAs in different ways [6, 7]. Strain and tissue differences in the inducibility of 2B1 and 2B2 mRNAs [8, 9] and species differences in the extent of PB induction [10–12] also suggest that more than one mechanism of induction may exist, or that the induction is modulated by distinct factors in various tissues and species.

One of the most striking species differences is seen in the effects of 1,4-bis-[2-(3,5-dichloropyridyloxy)]-benzene (TCPOBOP). This compound and some of its derivatives are extremely potent PB-like inducers in mice [13] yet are ineffective in rats [14]. In addition to the induction of the drug-metabolizing enzymes, both PB and TCPOBOP increase DNA synthesis and promote the development of tumours in mouse liver [15, 16]. The P450 induction by PB in mice is less studied than that in rats, but marked differences in the isozyme composition, catalytic properties and regulation in general seem to occur between these two species [10–12, 17–20]. In most of the previous studies with PB and TCPOBOP [21–23], only single dose levels were used and only monooxygenase activities were determined, leaving the response of individual P450 isozymes unknown. In the present study, we analysed the effect of TCPOBOP and PB on three mouse PB-responsive P450 isozymes [24], associated activities and mRNAs.

MATERIALS AND METHODS

Chemicals. The suppliers of substrates and reagents for electrophoresis, Western blotting and TLC

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‖ Abbreviations: BZDM, benzphetamine *N*-demethylase; BZDM/2C, BZDM inhibited by anti-P4502c-x IgG; COH, coumarin 7-hydroxylase; Cyt c red, NADPH cytochrome P450 reductase; PB, phenobarbital; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene; PROD, 7-pentoxyresorufin *O*-dealkylase; P450, cytochrome P450; SDS, sodium dodecyl sulphate; 1 × SSC = 150 mM NaCl, 15 mM Na-citrate, pH 7.0; 1 × Denhardt's = 0.02% each of bovine serum albumin, polyvinylpyrrolidine and Ficoll 400; TBS, 20 mM Tris–HCl + 500 mM NaCl, pH 7.6; TxαOH and TxβOH, testosterone α- and β-hydroxylation at carbon atom x.

¶ P450 *nomenclature:* P450 enzymes have been grouped, according to their nucleic acid sequence homology, into distinct families and subfamilies [47]. The trivial names P450Coh (coumarin 7-hydroxylase), P45015α (testosterone 15α-hydroxylase) and P450PBI are here replaced by the recommended names P4502a-5, P4502a-4 and P4502b-10, respectively [24, 32] (Honkakoski *et al.*, *Biochem J*, in press. Based on the N-terminal amino acid sequence [24], P450PBIII belongs to the subfamily 2C and it is most closely related to rat P4502C7. Due to lack of its full-length sequence, P450PBIII is assigned P4502c-x.

have been reported [24, 25]. 6β-, 7α- and 16α-hydroxytestosterone standards, and testosterone were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). PB was from University Pharmacy (Helsinki, Finland). TCPOBOP was a kind gift from Prof. Olavi Pelkonen, University of Oulu, Finland. The purity of TCPOBOP was more than 98% as judged from HPLC and MS analyses. 2α-, 15α- and 16β-hydroxytestosterone standards were provided by Prof. D. N. Kirk, MRC, London, U.K. [4-^{14}C]-Testosterone, [γ-^{32}P]ATP, [α-^{32}P]dCTP, Nytran sheets and Hyperfilm MP autoradiography films were from Amersham International (Amersham, U.K.). T4 polynucleotide kinase and oligolabelling kit were from Pharmacia (Uppsala, Sweden). All other chemicals were of at least analytical grade.

Preparation of enzymes and antibodies. Isozymes P4502a-5, P4502b-10 and P4502c-x were purified from pyrazole- or PB-treated D2 mouse liver, and polyclonal antibodies to these P450s were raised in rabbits [24, 26]. There were no cross-reactions between these antigens and respective antibodies in Western blotting assays and immunoinhibition experiments with reconstituted isozymes [24]. Anti-P4502a-5 IgG will react also with the 98.3% homologous P4502a-4 and the combined amounts (P4502a-4/5) are therefore presented. On Western blots, anti-P4502b-10 IgG recognizes its own 56-kDa antigen [24] and a related 53-kDa protein. The latter protein is well separated from P4502b-10 on SDS–PAGE gels, is not inducible by PB or TCPOBOP and we have identified it as the mouse P4502b-9*. A single band is detected by anti-P4502c-x IgG. These antibodies did not cross-react with any known mouse isozymes from subfamilies different from that of the antigen (Ref. 24 and unpublished results). NADPH P450 reductase (Cyt c red) was purified from the livers of PB-treated rats [27].

Treatment of animals and preparation of microsomes. Female DBA/2N/Kuo mice (D2; 8–9 weeks) were purchased from the National Laboratory Animal Center at the University of Kuopio and housed in macrolon cages. Standard rodent feed (Ewos, Sweden) and water were provided *ad lib.* The treatment groups consisted of three to five mice. The mice were given a single i.p. dose of TCPOBOP dissolved in olive oil. In the dose–response experiment, doses of 0, 0.03, 0.1, 0.3, 1.0, 3.0 or 10.0 mg/kg were given and the animals were killed 216 hr after dosage. In the time–response experiment, a dose of 3.0 mg/kg was given at 0, 1, 3, 8, 24, 72, or 216 hr before the animals were killed. PB was dissolved in saline. In the dose–response experiment, PB doses of 0, 3, 10, 30 or 100 mg/kg/day were given i.p. for 3 days. In the time–response experiment, a single PB dose of 100 mg/kg was given at 0, 1, 3, 8 or 24 hr. In the 72-hr group, two additional doses were given 24 hr apart. The animals were killed and the livers were divided into two parts. One part was frozen in liquid nitrogen and stored at −80° for the preparation of RNA, and the other was used for the preparation of microsomes [28].

Preparation of mRNA and hybridization to probes. Total RNA was prepared from the pooled frozen samples according to Chomczynski and Sacchi [29]. Poly-(A)-containing RNA (mRNA) was enriched with oligo-(dT)-cellulose [30]. mRNA was denatured and bound to Nytran filters with the slot-blot apparatus. The probes were labelled by using Pharmacia Oligolabelling Kit or T4 polynucleotide kinase and appropriate ^{32}P-labelled nucleotides. The hybridization to cDNA probes (sp. act. 0.5–2 × 10^9 cpm/μg) p15α-15 and pTF-1 was performed in 5 × SSC, 5 × Denhardt's, 0.1% sodium dodecyl sulphate (SDS) and 0.1 mg/mL sonicated salmon sperm DNA containing 50% formamide at 42°. The filters were washed with 1 × SSC, 0.1% SDS at room temperature (3 × 15 min) and 0.1 × SSC, 0.1% SDS at 55° (1–3 × 15 min) and then exposed to X-ray films. The hybridization to oligoprobe pf3/46-u4 (5′ GGAAGGTTGGCTCAACGA 3′ [31]; sp. act. 1 × 10^8 cpm/μg) was performed in 5 × SSC, 5 × Denhardt's, 0.1% SDS and 0.1 mg/mL sonicated salmon sperm DNA at 46°. The filters were washed with 2 × SSC, 0.1% SDS at 25° (2 × 15 min) and at 38° (2 × 15 min) and exposed to X-ray film. All signals were normalized to poly-(A)$^+$ RNA (mRNA) content on each slot detected by 5′-labelled oligo-(dT)$_{15}$ probe.

The cDNA probe p15α-15 will recognize both 2a-4 and 2a-5 mRNAs since the isozymes are 98.3% homologous at the amino acid level [32]. The probe pTF-1 codes for rat P4502C7, which is the closest relative to P4502c-x based on N-terminal amino acid sequences. Only one band is detected on Northern blots with mouse mRNA and pTF-1 probe [33]. However, other mRNAs from the 2C family, if present, could react with the probe [12]. The oligoprobe pf3/46-u4 [31] is specific for the 2b-10 mRNA.*

Monooxygenase assays. Microsomal protein content was measured by the bicinchonic acid method [34]. P450 content [35], NADPH cytochrome *c* reductase [36], coumarin 7-hydroxylase (COH) [26], 7-pentoxyresorufin *O*-dealkylase (PROD) [24], benzphetamine *N*-demethylase (BZDM) [24], and testosterone hydroxylase [36] activities were determined according to the methods cited. Immunoinhibition of BZDM, T16αOH and T16βOH activities was done with either preimmune IgG or saturating amounts of anti-P4502c-x or anti-P4502b-10 IgG (3 mg IgG/mg microsomal protein) added to microsomes 15 min prior to the initiation of the reaction with NADPH. P450 isozymes were reconstituted as before [24].

Western blotting. Electrophoresis was performed on 9% acrylamide gels [37] with 5–20 μg microsomal protein and 0.25–1 pmol of purified P450s as standards. The proteins were transferred on nitrocellulose sheets [38] with 12.5 mM Tris, 96 mM glycine and 20% methanol as transfer buffer. The sheets were then processed as described previously [24] with the exception that incubation times with the blocking agent and the primary antibody (1:500–1:2000 dilution) were reduced to 1 hr. After the colour development with 5-bromo-4-chloro-3-indolyl

* Honkakoski P, Kojo A and Lang MA, Regulation of mouse liver cytochrome P450 2B subfamily by sex hormones and phenobarbital. *Biochem J*, in press.

phosphate, the intensities of the bands were quantitated with a Shimadzu CS-9000 dual wavelength scanner.

Identification of the testosterone metabolites. Testosterone hydroxylation was carried out in 10-mL volumes with control and PB-induced microsomes to produce sufficient amounts of metabolites for analyses. The dichloromethane extract of assay mixture was evaporated, the residue was dissolved in acetone and the metabolites were chromatographed on TLC plates. The metabolites were identified under UV illumination by co-migration with authentic standards. The spots were extracted with dichloromethane and the solutions were dried under nitrogen stream. The standard substances and the extracted metabolites were trimethylsilylated with 50 μL of Sigma Sil A-reagent for 10 min at room temperature. The samples were then analysed on a HP 5890 gas chromatograph coupled to a VG Trio-2 quadrupole mass spectrometer. The metabolites were verified by comparing the retention times and the electron impact mass spectra with those of the standards. We found that the 15α-, 16α-, 7α-, 6β- and 16β-hydroxytestosterone spots were at least 90% pure and applicable for monooxygenase assays. An unidentified metabolite contaminated the 2α-hydroxytestosterone spot, which was not used in further studies.

Statistical methods. The analysis of variance with Scheffe's test was used to compare data between groups ($P < 0.05$ was considered statistically significant). When data was not distributed normally, a logarithm transformation preceded ANOVA. ED_{50} values and 95% confidence limits were calculated by probit analysis [39]. Pearson correlation coefficients were also calculated.

RESULTS

Marker enzymatic activities for mouse PB-responsive P450s

When monooxygenase activities from dose– and time–response experiments were correlated with corresponding P450 isozyme levels, we found good associations between P4502a-4/5, COH and T15αOH, between P4502b-10 and PROD, and between P4502c-x, T16βOH, and BZDM/2C (Table 1). These close correlations confirmed our previous studies [24, 32] showing that P4502b-10, P4502a-5 and P4502a-4 are responsible for the main part of the PROD, COH and T15αOH activities, respectively. They further suggested a role for P4502b-10 and P4502c-x in testosterone metabolism. When reconstituted, these isozymes could hydroxylate testosterone at positions 16α and 16β (turnover for P4502b-10 8.0 and 2.9 nmol/min/nmol P450, respectively). P4502c-x produced only the 16β-isomer (1.1 nmol/min/nmol P450) and an unidentified metabolite (1.3 nmol/min/nmol P450). Although P4502b-10 formed the 16β-isomer faster than P4502c-x, immunoinhibition studies (Fig. 1) revealed that at least 75% of T16βOH is inhibited by anti-P4502c-x IgG in control and PB-induced microsomes and only 10–15% by anti-P4502b-10 IgG. This points out that the principal isozyme for T16βOH belongs to different subfamilies in mice (2C) and rats (2B) [40].

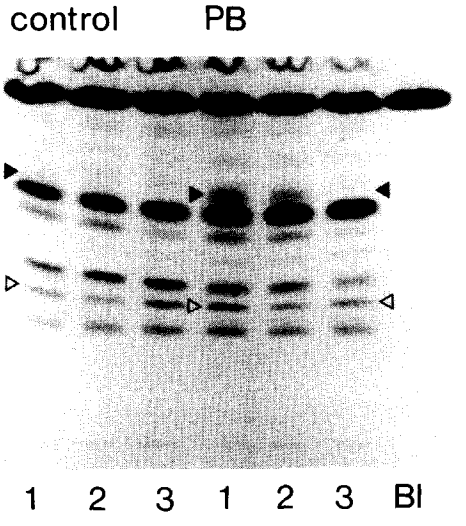

Fig. 1. The effect of anti-P4502b-10 and anti-P4502c-x IgG on testosterone hydroxylation. Immuno-inhibition of testosterone hydroxylation was done as described in Materials and Methods with control and PB-treated D2 mouse liver microsomes. Lanes 1, 2 and 3 contained equal amounts of preimmune, anti-P4502b-10 and anti-P4592c-x IgG, respectively. Bl denotes a blank reaction without microsomes. Spots corresponding to 16α- (▷) and 16β-hydroxytestosterone (▶) were scraped off and counted for radioactivity.

T16αOH was not affected by anti-P4502c-x IgG but it was inhibited 30% by the antibody against P4502b-10. Most of the T16αOH is mediated by isozyme(s) within the 2D family [18].

The extent and time-course of TCPOBOP and PB induction

The maximal induction varied dramatically from about a 3-fold increase in P4502a-4/5, related COH and T15αOH activities, and 2a-4/5 mRNA to more than a 50-fold induction of P4502b-10, PROD activity and associated mRNA content (Table 1 and Fig. 2). With low doses of TCPOBOP, P4502A-associated COH and T15αOH activities were even decreased (not shown). This might be caused by the vehicle, which might counteract the small elevating effects of low doses of inducer. A single dose of olive or corn oil decreased COH activity by 30–40% after 24 hr. However PROD, total BZDM, and BZDM/2C activities were not influenced by olive oil injections (not shown).

The time-course of induction (Fig. 3) revealed that P4502a-4/5 was increased 1.6-fold at 3 hr after the TCPOBOP injection with a parallel increase in COH activity, preceded by small increases in 2a-4/5 mRNA. Surprisingly, mRNA levels fell at 8 hr with a subsequent decline in protein and COH activity at 24 hr. The maximal induction was reached at 72 hr. In PB-treated animals, this biphasic effect was absent but the induction proceeded remarkably slowly as compared with isozymes P4502b-10 and P4502c-x. For the latter isozymes, mRNA and

Table 1. The maximal induction by TCPOBOP and PB of selected mouse monooxygenase activities and P450 isozymes, and correlation of P450 isozyme content to monooxygenase activities and mRNA levels in female D2 mice

	Specific activity and content*				ED_{50} (mg/kg)†		Correlation coefficient‡	
	Control	TCPOBOP	Control	PB	TCPOBOP	PB		
							P4502a-4/5 vs	
COH	229 ± 14	791 ± 47 (3.4)	267 ± 42	954 ± 96 (3.6)	0.45 (0.27–0.75)‖¶	34 (21–56)‖	COH	0.88
T15αOH	403 ± 43	857 ± 19 (2.1)	505 ± 59	1200 ± 148 (2.4)	0.61 (0.36–1.03)‖¶	33 (20–55)‖	T15αOH	0.77
P4502a-4/5	23 ± 1	52 ± 7 (2.3)	20 ± 2	54 ± 4 (2.7)	0.88 (0.54–1.42)‖¶	15 (9–26)	2a-4/5 mRNA	0.93
							P4502b-10 vs	
PROD	19 ± 1	2820 ± 485 (148)	20 ± 2	1967 ± 35 (98)	0.23 (0.13–0.42)‖	38 (25–60)‖¶	PROD	0.95
T16αOH	405 ± 41	1278 ± 46 (3.2)	454 ± 41	852 ± 33 (1.9)	0.16 (0.10–0.27)‖	23 (14–37)‖	T16αOH	0.75
P4502b-10	8 ± 2	404 ± 15 (50)	7 ± 1	358 ± 11 (51)	0.19 (0.10–0.39)‖	21 (12–37)‖	2b-10 mRNA	0.88
							P4502c-x vs	
BZDM/2c§	4.5 ± 0.1	18.2 ± 0.2 (4.1)	4.5 ± 0.5	22.6 ± 2.8 (5.0)	0.04 (0.03–0.06)	10 (6–18)	BZDM/2C	0.89
T16βOH§	340 ± 19	1461 ± 147 (4.3)	346 ± 46	1770 ± 101 (5.1)	0.06 (0.03–0.11)	9 (6–16)	T16βOH	0.91
P4502c-x§	50 ± 3	216 ± 13 (4.3)	42 ± 4	228 ± 18 (5.4)	0.03 (0.02–0.04)	5 (3–9)	pTF-1 mRNA	0.89

* P450 isozyme content is expressed as pmol/mg protein, and monooxygenase activities as pmol/min/mg protein except BZDM/2C (nmol/min/mg protein). Data are means ± SD from 3–5 animals. Maximal-fold induction is given in parentheses.

† ED_{50} values were calculated [39] and 95% confidence limits are given in parentheses. The superscripts indicate a statistically significant difference from P4502C‖ or related activities.¶

‡ Correlations between P450 isozymes and monooxygenase activities were calculated from dose– and time–response experiments ($N \geq 75$), and correlation to mRNA levels was determined using the dose–response experiments only ($N = 12$).

§ The *maximal* induction of BZDM/2C, T16βOH and P4502c-x occurs already at 0.1 mg/kg TCPOBOP in contrast to other isozymes (3–10 mg/kg). At 10 mg/kg, the results were: BZDM/2C, 8.7 ± 1.6 nmol/min/mg; T16βOH, 1355 ± 56 pmol/min/mg; and P4502c-x, 144 ± 16 pmol/mg.

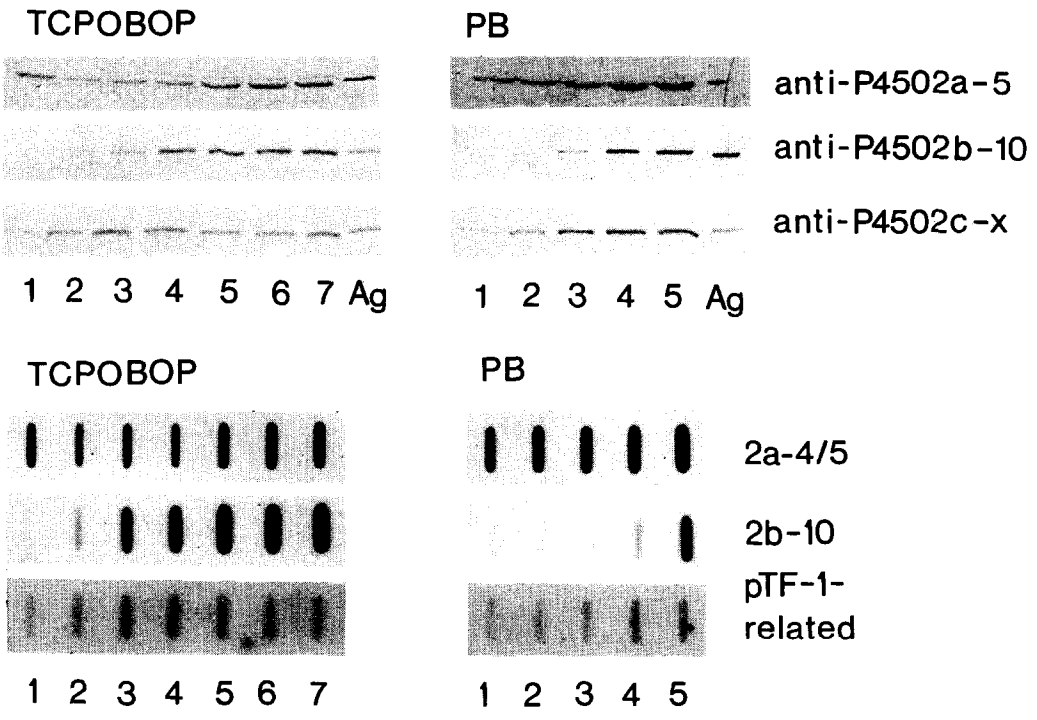

Fig. 2. Dose-dependent induction of P450 isozymes and mRNAs by TCPOBOP and PB. The dose–responses of P450 isozymes (upper panels) and associated mRNAs (lower panels) to TCPOBOP (left panels) and PB (right panels) were quantitated as described in Materials and Methods. In the left panels, TCPOBOP doses of 0, 0.03, 0.1, 0.3, 1, 3 and 10 mg/kg correspond to lanes 1–7. In the right panels, PB doses of 0, 3, 10, 30 and 100 mg/kg correspond to lanes 1–5. Ag denotes 0.25–1.0 pmol of indicated antigen. For detection of P4502c-x and P4502a-4/5, 5 and 15 μg of microsomal protein were used, respectively. For P4502b-10 5 μg were used with the exception of 20 μg for dose group 0 mg/kg (upper panels, lane 1). Results from one determination containing individual microsomes are shown. Serial dilutions of isolated poly-(A)$^+$ RNA were probed with indicated oligonucleotide or cDNA probes. For clarity, results from 2 μg RNA loading are shown.

protein were increased already at 3–8 hr after the injection of inducers.

The extent of the increase in both P450 isozyme content and mRNA levels were very similar and correlated well in dose–response experiments (Table 1 and Fig. 2). This suggests that the induction of P450s by TCPOBOP AND PB is controlled primarily at pretranslational levels. PB could modulate the expression of P4502b-10 and P4502c-x also (post-)translationally, since the protein levels were increased somewhat more (30–50%) than the mRNA levels. However, in the case of pTF-1 probe, several (differentially regulated ?) 2C mRNAs might be recognized [12, 33].

Differential inducibility of P450s *by TCPOBOP and PB*

The quantitative and temporal differences in induction of individual P450s suggest that different pathways or modulators for induction might exist. When the dose–response data was analysed (Table 1), we found that the ED_{50} value for P4502c-x and associated activities was about 0.03 mg/kg TCPOBOP, which was 6–30 times less than the ED_{50} values for P4502b-10, P4502a-4/5 and associated activities. With PB, the distinction between P4502c-x and the other isozymes was less pronounced but still evident: the ED_{50} for P4502c-x induction was three to four times smaller than that for P4502b-10, P4502a-4/5, and associated activities. Other monooxygenase activities measured (T6βOH, T7αOH, BZDM, Cyt c red, P450; not shown) also differed from P4502c-x-related parameters.

Slight differences in the maximal induction elicited by PB and TCPOBOP were observed: PB tended to increase 2A-related T15αOH and COH and 2C-related T16βOH and BZDM/2C more than TCPOBOP, whereas the reverse occurred with T16αOH and PROD (Table 1). Furthermore, at high doses of TCPOBOP (3–10 mg/kg) where isozymes P4502b-10 and P4502a-4/5 were maximally induced, the content of P4502c-x was decreased to submaximal levels (Table 1 and Fig. 2). This was not the case with highest dose of PB. Although not conclusive, these data might indicate that PB and TCPOBOP induction involves at least partially different factors. No significant differences between PB and TCPOBOP were seen in the induction of other parameters measured (T6βOH, T7αOH, BZDM, Cyt c red, P450; not shown).

DISCUSSION

To our knowledge, this is the first report in which

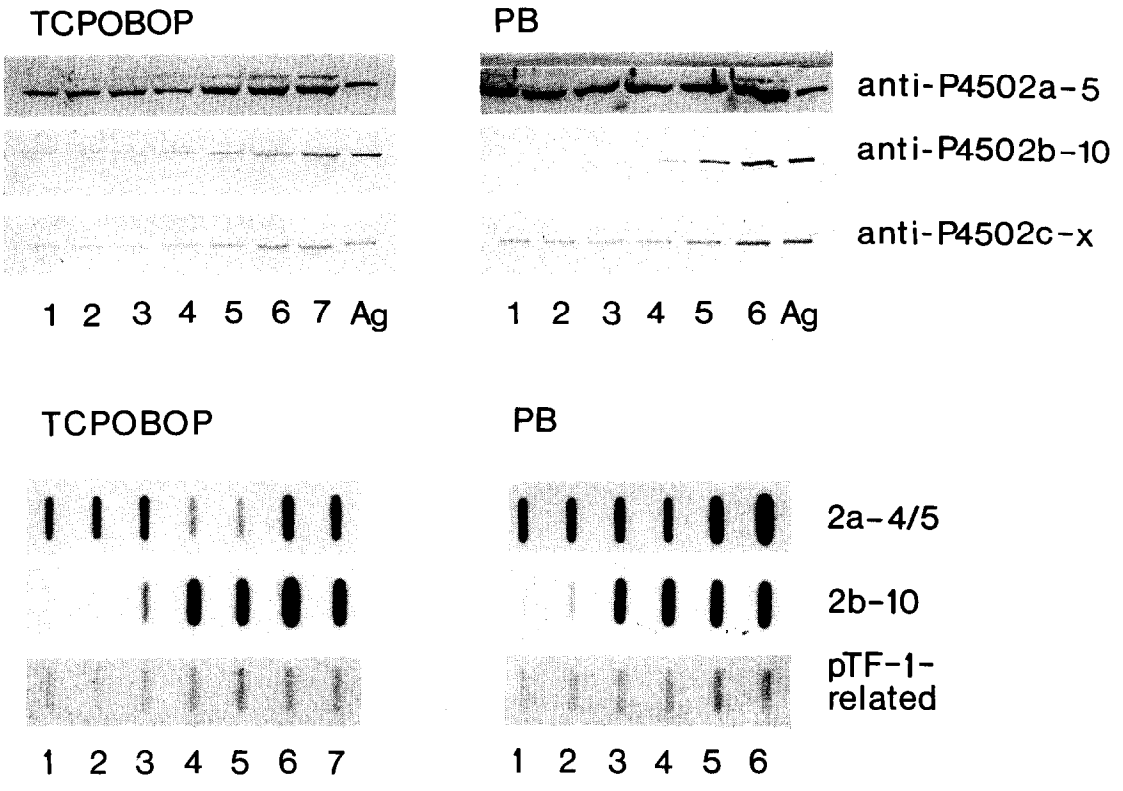

Fig. 3. Time-dependent induction of P450 isozymes and mRNAs by TCPOBOP and PB. The time-responses of P450 isozymes (upper panels) and associated mRNAs (lower panels) to induction by TCPOBOP (left panels) and PB (right panels) were quantitated as described in Materials and Methods. Times 0, 1, 3, 8, 24, 72 hr after injection correspond to lanes 1–6. Lane 7 (TCPOBOP only) corresponds to time 216 hr. All other symbols are as in Fig. 2. Protein amounts were as in Fig. 2 except for P4502b-10 (20 μg for lanes 1–4, 5 μg for other lanes). Results from 2 μg RNA loading are shown.

the effects of PB and especially TCPOBOP on mouse P450 isozymes and mRNAs have been analysed, contrary to other activity-based studies. The reported lack of induction of T15αOH by a single dose of TCPOBOP [22] could not be confirmed: we observed a time- and dose-dependent increase in 2A-related activities, proteins and mRNA in TCPOBOP-treated D2 mice. Based on various monooxygenase assays, it was concluded that TCPOBOP is less specific than PB as an inducer [23]. The interpretation of this study is difficult given the fact that for most of the substrates used, a major catalyst within the mouse P450s is not known, and that multiple differentially regulated isozymes might catalyse the reactions measured. High doses of TCPOBOP but not PB diminish the expression of P4502c-x to submaximal levels, explaining the previously found difference between the inducers [24]. Since P450b-10 and P4502c-x catalyse N-demethylation reactions efficiently [24] and also participate in testosterone metabolism, their differential inducibility could underlie some of the results in the above-mentioned studies.

Perhaps the most significant finding was the differential sensitivity of the isozymes to the inducers: P4502c-x, a member of the P450 2C subfamily, and associated T16βOH and BZDM/2C activities are induced by much lower doses of PB and TCPOBOP than the isozymes from subfamilies 2A and 2B. This distinction was also reflected in the mRNA levels suggesting that the inducers may regulate P450 expression via different pathways but at the mRNA level. While these experiments were in progress, it was reported that rat mRNAs from the different gene families 3A and 2B differ in their sensitivity to PB [7]. The major PB-inducible mRNAs 2B1 and 2B2 were regulated coordinately [7]. These findings support our data which suggest that, even within the same gene family, more than one pathway for PB induction might exist. The factors controlling the induction by PB and TCPOBOP are largely unknown although the transcription rate of rat 2B genes is increased after PB administration [3, 41]. Since TCPOBOP is the most potent PB-like inducer, it may be helpful in the clarification of induction mechanisms.

PB and TCPOBOP appear to control 2a-4/5 mRNA and protein coordinately. On the other hand, pyrazole induces this mRNA to disproportionally high levels when compared to COH or T15αOH

activity [42]. No increase in gene transcription by pyrazole was detected* and later studies have suggested that pyrazole might stabilize the mRNA [43]. This indicates that at least two distinct mechanisms of regulation control P4502a-4/5 expression. Since neither the antibody nor the cDNA probe discriminates between the isozymes, they might still be differently regulated. However, by using a diagnostic restriction enzyme digestion [44], no change in the ratio of 2a-4 to 2a-5 mRNA was found after PB treatment.* Approximation of isozyme levels from microsomal activities (Table 1) and from turnover numbers of purified P450s (85 nmol/min/nmol P4502a-4, 20 nmol/min/nmol P4502a-5 [24, 45]) indicate that P4502a-4 is increased from 6 to 14 pmol/mg and P4502a-5 from 14 to 49 pmol/mg by PB. The ratio of the isozymes is quite similar in control and PB-induced microsomes, and their sums (20 and 63 pmol/mg) coincide reasonably well with the observed values of 20 ± 2 and 54 ± 4 pmol/mg.

The interesting biphasic time response of 2A-related parameters to TCPOBOP is difficult to explain. It may be related to the effects of the vehicle, or perhaps to the stabilization by TCPOBOP of pre-existing 2a-4/5 mRNA. In any case, the early changes are regulated pretranslationally. This resembles the situation after pyrazole administration when COH activity had already risen at 3 hr and was maintained at that level for the next 9 hr. After that, the increase in COH started to proceed to maximal levels.* The time-course of the increase in microsomal epoxide hydrolase by TCPOBOP is also biphasic [46] with a transient rise at 4 hr and a return to control values at 20 hr, before maximal induction after several days.

In conclusion, we have shown that mouse P450 isozymes, belonging to subfamilies 2A, 2B and 2C, each have distinct patterns of dose- and time-response to PB and TCPOBOP. The expression of specific isozymes parallels the levels of corresponding mRNAs well, suggesting that pretranslational events are the major factors controlling induction. Testosterone 16β-hydroxylation activity serves as a good marker activity for the mouse P4502c-x.

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