1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene, an extremely potent modulator of mouse hepatic cytochrome *P*-450 gene expression

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We have investigated the effects of TCPOBOP {1,4-bis[2-(3,5-dichloropyridyloxy)]benzene}, a potent cytochrome *P*-450-inducing agent [Poland, Mak, Glover, Boatman, Ebetino and Kende (1980) *Mol. Pharmacol.* **18**, 571–580], on cytochrome *P*-450 isoenzyme expression in the mouse. Hepatic cytochrome *P*-450s from several distinct gene families were strikingly induced by a single dose of 75 µg of the compound. Northern-blot analysis demonstrated that this induction was almost certainly due to transcriptional activation of the cytochrome *P*-450 genes. The potency of this inductive effect was further reflected in the finding that cytochrome *P*-450 levels were still increased 12 weeks after a single injection of 75 µg of this compound. Interestingly, the mRNA levels of certain other genes, including those of metallothionein and the mouse major urinary proteins, were also

induced. In view of the similarity in the effects of TCPOBOP and the synthetic glucocorticoid dexamethasone on mouse hepatic gene expression, we determined whether TCPOBOP acts through the glucocorticoid receptor. This did not, however, appear to be the case. Experiments with hypophysectomized animals demonstrated that TCPOBOP action was not regulated indirectly via the pituitary. In addition, induction of mouse Cyp2b protein by TCPOBOP in a primary culture of mouse hepatocytes suggests that the compound has a direct action on mouse liver. The above findings demonstrate that TCPOBOP is one of the most potent modulators of cytochrome *P*-450 gene expression described to date. It is not inconceivable that a single dose of this compound may alter hepatic gene expression for the majority of the lifespan of a mouse.

INTRODUCTION

1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (Figure 1), which was first isolated as a pesticide contaminant, has been shown to be an extremely potent phenobarbital-like inducer of mouse hepatic cytochrome *P*-450-dependent mono-oxygenase activity (Poland et al., 1980). This was reflected both in the low dose of the compound required for induction and the resulting longevity of the observed inductive effects. A single dose of TCPOBOP was found to induce mono-oxygenase activity for more than 20 weeks (Poland et al., 1980).

Intriguingly, TCPOBOP is species-specific in its effects. For example, it does not increase cytochrome *P*-450 levels in rats or guinea pigs, even at substantially higher doses (Poland et al., 1981; Romano et al., 1986), but does cause induction in Syrian golden hamsters. It has been speculated that this species difference may be due to the presence or absence of a TCPOBOP-responsive receptor in mice and rats respectively (Romano et al., 1986). Structure–activity studies led Kende et al. (1985) and Kelley et al. (1985) to propose the existence of a relatively specific receptor for bis-pyridyloxybenzene congeners, with two similar and complementary binding sites. However, no such receptor has as yet been identified.

In addition to its capacity to induce mono-oxygenase activity, TCPOBOP has a variety of further effects on mouse liver. In particular, like phenobarbital, it causes a profound and rapid increase in liver size. Probably as a consequence of this effect, TCPOBOP has been shown to be a tumour promoter of diethyl-

nitrosamine-induced hepatocarcinogenesis (Dragani et al., 1987; Della Porta et al., 1987).

It was the aim of work described here to further characterize and evaluate the mechanism of TCPOBOP action, as this may throw new light on both the mechanisms of cytochrome *P*-450 regulation and the control of liver growth. Since the effects of this compound were first reported, there has been an enormous increase in our knowledge of the cytochrome *P*-450-dependent mono-oxygenase system in mammals and it is now known that this superfamily of proteins comprises more than ten families (Nebert et al., 1991). Many cytochrome *P*-450 genes are inducible at the level of transcription by different xenobiotics. For example, the CYP1A family is induced by polycyclic aromatic hydrocarbons and the CYP2B family by phenobarbital, a process that appears to have evolved as an adaptive response to environmental challenge (Wolf, 1991). The evolution, regulation and molecular genetics of the cytochrome *P*-450 supergene family have been extensively reviewed (Gonzalez, 1990; Gonzalez and Nebert, 1990; Henderson and Wolf, 1992).

Although TCPOBOP has been shown to induce microsomal mono-oxygenase activity, it is not known which cytochrome *P*-450 proteins are affected. Also, it has not previously been established whether these effects occur at a transcriptional level. Intriguingly, the synthetic glucocorticoid dexamethasone demonstrates a similar species difference in the induction of cytochrome *P*-450s to that observed for TCPOBOP (Meehan et al., 1988). This raised the possibility that these compounds may act via the same mechanism, possibly through the glucocorticoid receptor.

Abbreviations used: TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; MT-II, metallothionein-IIa; MUPS, major urinary protein; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GRE, glucocorticoid-responsive promoter element.

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Figure 1 Structure of TCPOBOP

This possibility was investigated in the experiments described below.

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, all chemicals were purchased from either Sigma Chemical Co., Poole, Dorset, U.K. or BDH, Glasgow, U.K., and were of analytical grade or better. Nitrocellulose was obtained from Schleicher and Schüll, Dassel, Germany, and ^{125}I -Protein A and $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ from Amersham International, Amersham, Bucks., U.K. Resorufin analogues were purchased from Molecular Probes Inc., Junction City, OR, U.S.A.

Synthesis of TCPOBOP

TCPOBOP was synthesized by a modified version of the method of Poland et al. (1980). The final product was analysed by n.m.r. spectroscopy and m.s. to confirm the chemical structure and molecular mass. TCPOBOP used in initial experiments was a generous gift from Dr. Alan Poland and Dr. Andrew Kende.

Animal treatment

Adult male and female DBA/2 mice were obtained from Banton and Kingman and were fed *ad libitum* on standard animal diet. The mice (ten of each sex per treatment group) were treated intraperitoneally with the cytochrome *P*-450-inducing agents sodium phenobarbital (80 mg/kg per day for 3 days in phosphate-buffered saline) or dexamethasone (100 mg/kg per day for 4 days in corn oil). A further group of mice were given a single injection of TCPOBOP at 3 mg/kg, intraperitoneally in corn oil, and killed after 4 days, 2 weeks, 4 weeks, 8 weeks or 12 weeks. Control animals received corn oil only.

After the last day of treatment, or on day 4 for the TCPOBOP-treated group, the animals were overnight before being killed by cervical dislocation. The organs were removed rapidly, rinsed in phosphate-buffered saline, and frozen on solid CO_2 . Tissue was stored at -70°C before processing.

C57BL/6 mice were obtained from Charles Rivers, where parapharyngeal hypophysectomy and sham operations were carried out. The extent of hypophysectomy was assessed by monitoring weight gain of the hypophysectomized animals relative to the sham-operated controls. Both groups were then treated intraperitoneally with a range of cytochrome *P*-450-inducing agents as described above.

Preparation of microsomes

Microsomal samples were prepared from liver, lungs, kidneys and testes by differential centrifugation from pooled tissue, as previously described (Meehan et al., 1988). Microsomes were finally resuspended in a solution containing 10 mM potassium phosphate buffer, pH 7.4, 1% (w/v) KCl, 1 mM EDTA and 0.25 M sucrose, and stored at a concentration of 10–20 mg/ml at -40°C .

Protein content was determined by the method of Lowry et al. (1951), with BSA as standard.

Cytochrome *P*-450 substrate metabolism

The oxidative metabolism of the alkoxyphenoxazone (resorufin) analogues (benzyloxy-, pentoxy-, ethoxy- and methoxy-) by cytochrome *P*-450 was determined fluorimetrically by measuring the rate of generation of fluorescent product (Burke et al., 1985).

Immunoblotting

SDS/PAGE was carried out by the method of Laemmli (1970), using 9% separating gels.

Immunoblotting was performed essentially as described by Towbin et al. (1979), with modifications as described by Lewis et al. (1988). After separation, proteins were transferred electrophoretically to nitrocellulose, and probed with polyclonal antisera to various rat liver cytochrome *P*-450s. Antibodies to the purified enzymes were isolated as described previously (Wolf et al., 1984, 1986). These antibodies have previously been extensively used in immunoblotting studies with mouse microsomal samples (Meehan et al., 1988; Henderson et al., 1990). In addition, the isoenzyme specificities of the antisera have been demonstrated by immunoblot analysis with expressed human recombinant cytochrome *P*-450 proteins (Forrester et al., 1992). Cytochrome *P*450 CYP4A1 was isolated by the method of Tamburini et al. (1984), and was of high purity, running as a single band on SDS/PAGE. Antibodies used were to CYP1A1, CYP2A1, CYP2B1, CYP2C6, CYP2E1 (a gift from Dr. C. S. Yang), CYP3A1 and CYP4A1. [The nomenclature system used throughout this manuscript is that of Nebert et al. (1991).]

After visualization of the immunoreactive polypeptides with horseradish-peroxidase-labelled second antibody and 4-chloro-1-naphthol as substrate, the signal was enhanced with ^{125}I -Protein A (Amersham International) and subsequent autoradiography (Kodak X-Omat AR5 X-ray film) with intensifying screens at -70°C . Differing exposure times were used in order to optimize the autoradiographic signal from each blot.

In order to determine the relative levels of cytochrome *P*-450 expression, an appropriate exposure of each autoradiograph was scanned using a Joyce-Loebl scanning densitometer. Linearity of the method was established by generating a standard curve from a series of microsomal standards loaded in serial dilutions.

Northern blotting

mRNA was isolated from liver samples of male and female DBA/2 mice as previously described by Cox (1968). RNA concentration and purity was estimated spectrophotometrically, and tested for integrity by ethidium bromide staining. mRNA was separated on denaturing formaldehyde gels using hybridization conditions described previously (Meehan et al., 1984). After transfer to Hybond-N, the RNA was cross-linked to the membrane by u.v. irradiation. Blots were washed at 65°C with solution containing 0.3 M NaCl, 0.03 M trisodium citrate, pH 7.4, 0.1% SDS and 0.1% sodium pyrophosphate. cDNA probes were labelled by random priming (Rigby et al., 1977; Feinberg and Vogelstein, 1983, 1984).

Human cDNA probes used were: CYP1A (1088 bp), CYP2A6 (1.75 kb), CYP2E1 (1.6 kb) and CYP3A4 (727 bp). The rat CYP2B1 (1.7 kb) and CYP2C6 (1.4 kb) cDNA probes were kindly provided by Dr. M. Adesnik, and the rat CYP4A1 (2.1 kb) probe was from Dr. G. G. Gibson. All probes were shown to be specific for their respective gene families in mouse by their pattern of hybridization and by mapping in recombinant inbred

lines (Miles et al., 1991). Equivalence of RNA loading was monitored by probing with a mouse actin cDNA probe (1.2 kb), a gift from Dr. Joan McNab. The mouse metallothionein IIa (MT-II) cDNA probe (220 bp) was kindly provided by Dr. Peter Searle and the mouse major urinary protein (MUPS) cDNA probe (714 bp) by Dr. Reya Al-Shawi.

Cell culture and transfection

ZR-75 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing Phenol Red with 10% (v/v) fetal calf serum (FCS) (Gibco, Bethesda Research Laboratories, MD, U.S.A.) and 10 nM oestradiol. For transfection, cells were plated at 5×10^5 per 6 cm dish in Phenol-Red-free DMEM containing 5% (v/v) FCS from which endogenous steroids had been removed by treatment with dextran-coated charcoal. Transient transfections were performed using the calcium phosphate precipitation technique (Wigler et al., 1979), essentially as described by Ham et al. (1988). The cells were then maintained for 48 h in the appropriate hormones. Dexamethasone and R5020 were added to 0.1 μ M, and phenobarbital and TCPOBOP to a range of concentrations from 1 μ M to 1 nM. Chloramphenicol acetyltransferase (CAT) activity was assayed non-chromatographically (Sleigh, 1986) and normalized for transfection efficiency with luciferase activity.

The construction of the reporter plasmid pLTR-CAT has been described previously (Parker et al., 1987). The control vector pJ3 Luciferase consists of the luciferase gene (De Wet et al., 1987) cloned into the eukaryotic expression vector pJ3 Ω (Morgenstern and Land, 1990).

Hepatocyte isolation and culture

Hepatocytes were isolated from adult male Alderley Park Swiss mice (20–25 g) by *in situ* perfusion of the liver with collagenase essentially as described by Mitchell et al. (1984), with the following modifications: the superior vena cava was cut and the perfusate was not recirculated; flow rate was 12 ml/min and collagenase digestion was 50 mg of collagenase/250 ml of perfusate for 10 min. The viability of isolated hepatocytes was determined by Trypan Blue dye exclusion and was greater than 80%. Hepatocytes isolated from the two animals were pooled, and primary monolayer cultures were prepared by seeding 2×10^6 viable cells in 4 ml of CL15 medium in 25 cm² tissue culture flasks as described by Mitchell et al. (1984). At 4 h after seeding, the spent medium was aspirated and fresh medium applied. Phenobarbital dissolved in 0.9% NaCl and TCPOBOP dissolved in dimethylformamide were added to the medium when it was changed each day (at 24, 48 and 72 h after seeding). Hepatocytes were therefore exposed for 3 days to 2 mM phenobarbital, 2, 10 or 50 μ M TCPOBOP or vehicle alone (1.5 mM NaCl or 0.25% dimethylformamide). Observation under a phase-contrast microscope showed the presence of a few or numerous crystal needles in the culture medium containing 10 or 50 μ M TCPOBOP respectively, indicating the limit of solubility of this compound in CL15 medium. At 96 h after seeding, the cells from individual flasks were harvested in 1 ml of SET buffer [0.25 M sucrose/5 mM EDTA/20 mM Tris/HCl (pH 7.4)] and were then disrupted by sonication.

Purified rat cytochrome *P*-450 CYP2B1 and CYP2B2 (0.8 pmol) and duplicate samples of hepatocyte sonicates (85 μ g of total cellular protein) were separated by SDS/PAGE using 7.5% (w/v) separating gels. Each sample of hepatocyte sonicates contained pooled cells from two individual flasks. Immunoblotting with rat cytochrome *P*-450 CYP2B1 antisera was performed as described by Bars et al. (1989).

RESULTS

Initial experiments were carried out to determine the cytochrome *P*-450 enzymes induced by TCPOBOP in mouse liver (Figure 2). For comparison, liver microsomes from animals treated with dexamethasone and phenobarbital were included.

With the exception of Cyp2e1, the levels of protein from all the cytochrome *P*-450 families and subfamilies examined were significantly increased after a single injection of 75 μ g of TCPOBOP. Many similarities could be seen with the induction pattern observed for phenobarbital. The relatively poor characterization of mouse cytochrome *P*-450 forms at the protein level does not allow the exact identity of some of the proteins to be assigned. Also, in certain cases, it cannot be ruled out that the antibody recognizes more than one cytochrome *P*-450 with the same mobility.

The inductive effects of TCPOBOP appear to fall into three categories. (1) There was little or no effect in the case of Cyp2e1 (the upper band on the blot shown in Figure 2). This band was identified as Cyp2e1 on the basis of the relative mobility of the recombinant mouse Cyp2e1 protein (J. E. Freeman and C. R. Wolf, unpublished work). We believe that the lower band recognized by the antibody to Cyp2e is Cyp2b9 protein (Freeman et al., 1992). (2) There was some induction (2–5-fold) in the case of Cyp2c, Cyp3a and Cyp4a. (3) There was a very profound increase in the levels of Cyp1a, Cyp2a and Cyp2b proteins. TCPOBOP had no effect on cytochrome *P*-450 isoenzyme expression in kidney, lung and testes (results not shown).

On the basis of measurement of the rate of metabolism of a series of resorufin analogues (Table 1), the hepatic Cyp1a protein induced by TCPOBOP appears to be Cyp1a2, as the activity

Figure 2 Effect of TCPOBOP on mouse hepatic cytochrome *P*-450 levels

Male (M) and female (F) DBA/2 mice were treated with dexamethasone (Dex), phenobarbital (PB) or TCPOBOP. Liver microsomal samples (15 μ g) from these animals, or suitable controls, were separated by SDS/PAGE (9% gels) and cytochrome *P*-450 isoenzyme content was determined by Western-blot analysis using various cytochrome *P*-450 antisera as detailed in the Materials and methods section. S, purified rat cytochrome *P*-450 standards.

Table 1 Effect of TCPOBOP on cytochrome *P*-450 substrate metabolism

The metabolism of a series of resorufin analogues was determined using liver microsomes from male (M) and female (F) DBA/2 mice treated with dexamethasone (Dex), phenobarbital (PB) or TCPOBOP (TC). Experimental details are described in the Materials and methods section. Each experiment was done in triplicate.

1. Benzyloxyresorufin metabolism			
Treatment group	Specific activity of resorufin production (pmol/min per mg of microsomal protein)		
Control (F)	4.26		
Control (F)	0.50		
Dex (M)	2.30		
Dex (F)	177.19		
PB (M)	13.26		
PB (F)	286.09		
TC (M)	79.86		
TC (F)	540.00		
2. Alkoxyresorufin metabolism			
	Specific activity of resorufin production (pmol/min per mg of microsomal protein)		
Treatment group	Methoxy-	Ethoxy-	Pentoxy-
Control (M)	3.10	2.70	5.20
Control (F)	3.30	2.80	5.00
TC (M)	14.17	5.30	8.90
TC (F)	62.09	23.20	21.99

towards 7-ethoxyresorufin, a substrate for Cyp1a1 (Burke et al., 1985), was only slightly increased (6-fold). This induction of Cyp1a protein by both phenobarbital and TCPOBOP is interesting, and has not previously been reported for this class of compound. The Cyp2b protein induced may be that described by Noshiro et al. (1988) as the major phenobarbital-inducible enzyme, Cyp2b9. This enzyme is sexually dimorphic in mice, and appears to be induced to a greater degree in females than in males (Meehan et al., 1988). Marked sexual differentiation in the expression of this protein is substantiated by the induction of benzyloxyresorufin activity (see Table 1). However, the sexual differentiation in the induction of Cyp2b protein was less marked on Western-blot analysis (Figure 2). The reason for this is unclear but may be due to induction of further Cyp2b proteins. Similarly, cytochrome *P*-450s in the Cyp2a gene family are probably those described as Cyp2a4 and Cyp2a5 (the upper and lower bands respectively) (Squires and Negishi, 1988). Cytochrome *P*-450s in the Cyp2c, Cyp3a and Cyp4a gene families have not been characterized in the mouse. At least two proteins within the Cyp3a gene family appear to be TCPOBOP-inducible. Whereas phenobarbital induced at least three distinct proteins in the Cyp4a gene family, TCPOBOP administration caused significant induction of only one of these proteins. The assignment of these proteins to the Cyp4a gene family was confirmed using monoclonal antibodies to rat CYP4A proteins (C. J. Henderson, T. Bammler, C. Savoy, F. Waechter and C. R. Wolf, unpublished work).

In agreement with our previous study, dexamethasone was a potent inducer of Cyp2b. However, in contrast with our previous report (Meehan et al., 1988), the levels of Cyp2c and Cyp3a proteins were not significantly increased by the compound in the experiments shown. This appears to be due to the lower dose of dexamethasone administered (100 compared with 200 mg/kg) or the mouse strain used (C57/BL6 compared with DBA/2) (see

Figure 3 Effect of TCPOBOP on cytochrome *P*-450 mRNA levels

Liver RNA (10 µg) from male and female DBA/2 mice from controls or animals treated with dexamethasone (Dex), phenobarbital (PB) or TCPOBOP was separated on 1% denaturing formaldehyde/agarose gels, transferred overnight to Hybond-N, and hybridized with cytochrome *P*-450 cDNA probes as detailed in the Materials and methods section.

Figure 6). mRNA levels encoded by the Cyp2c and Cyp3a genes were, however, elevated on dexamethasone treatment.

The effect of TCPOBOP, dexamethasone and phenobarbital on cytochrome *P*-450 mRNA levels in most cases paralleled the changes in protein expression extremely closely (Figure 3), the only exceptions being the induction of Cyp2c and Cyp3a mRNAs by dexamethasone without concomitant changes observed in the level of protein expression. Also, there appeared to be no correlation between the levels of Cyp4a mRNA and expressed protein. This may be due to the complexity of this gene family in the mouse and the possibility that the cDNA probe used in the Northern-blot analysis hybridizes specifically to one mRNA species. There was an interesting similarity between TCPOBOP and dexamethasone, but not phenobarbital, in the marked induction of a male-predominant Cyp4a mRNA.

The main conclusion from the Northern-blot analysis, therefore, was that after TCPOBOP administration, mRNA levels encoded by genes from almost all the individual cytochrome *P*-450 gene families were increased, indicating that the changes in protein expression were due to an increased rate of transcription. However, the possibility of mRNA stabilization cannot be ruled out at the present time. In most cases, no sexual differentiation in the ability of TCPOBOP to induce cytochrome *P*-450 mRNA was observed.

Figure 4 Long-term induction of cytochrome *P*-450 proteins by TCPOBOP

Liver microsomes (15 μ g) from male (M) and female (F) DBA/2 mice, treated with a single injection of TCPOBOP were separated by SDS/PAGE (9% gels), transferred to nitrocellulose and probed with various cytochrome *P*-450 antisera, as described in the Materials and Methods section. Samples were prepared 2, 4, 8 and 12 weeks after TCPOBOP administration. S, rat cytochrome *P*-450 standard. The reason why no bands are seen for Cyp2e at 12 weeks is unclear.

Table 2 Effect of TCPOBOP and phenobarbital on the activation of the long terminal repeat

CAT activity was measured after transfection of the murine mammary tumour virus long terminal repeat fused to CAT into ZR-75 cells and subsequent treatment with a range of inducing agents. Mock, mock transfection; —H, transfection without the addition of an inducing agent; R5020, a potent synthetic glucocorticoid; Dex, dexamethasone; PB, phenobarbital; TC, TCPOBOP. 'Normalized activity' is corrected for transfection efficiency by co-transfection with luciferase, i.e. column 1/column 2.

Treatment	CAT activity (c.p.m.)	Luciferase activity (c.p.m.)	Normalized CAT activity (c.p.m.)
Mock	4304	2.87	1501.75
—H	1473.5	82.36	17.89
Dex	98770	114.40	863.37
R5020	134320	121.90	1101.89
PB 1 μ M	4089	83.55	48.94
0.1 μ M	1728	60.30	28.66
10 nM	1575	61.85	25.47
1 nM	1410	84.85	16.62
TC 1 μ M	537	95.35	5.63
0.1 μ M	1103	98.9	11.15
10 nM	1270.5	82.85	15.34
1 nM	1193.5	75.45	15.82

In order to establish the potency of TCPOBOP as a cytochrome *P*-450 inducer, liver samples were taken for a period of up to 3 months after a single injection of 3 mg/kg of the compound.

Figure 5 Analysis of MT-II and MUPS mRNA levels after TCPOBOP treatment

The same mRNA samples as used for the cytochrome *P*-450 analysis were used. Hybridization conditions were as described for cytochrome *P*-450 analysis. (a) Short-term experiment; (b) long-term experiment.

Figure 6 Effect of hypophysectomy on TCPOBOP induction of cytochrome *P*-450 proteins

Male (M) and female (F) C57BL/6 mice were hypophysectomized or sham-operated as described in the Materials and methods section, before being treated with dexamethasone (Dex), phenobarbital (PB) or TCPOBOP. Liver microsomal samples from these animals (15 μ g) were separated by SDS/PAGE using 9% gels, and cytochrome *P*-450 isoenzyme content was determined by Western-blot analysis using antisera to the cytochrome *P*-450 isoenzymes shown. Hx, hypophysectomized; Sh, sham-operated; Con, control; S, purified rat cytochrome *P*-450 standard.

These experiments (Figure 4) clearly confirmed the ability of TCPOBOP to induce the expression of cytochrome *P*-450s from almost all the gene families studied and also demonstrated that the induction was long lasting. Although, in some cases, the level of induction at 12 weeks was decreased relative to that observed

after 2 weeks, in others only a slight decrease was seen. In more recent studies, we have shown that cytochrome *P*-450 levels remain highly induced for at least 6 months after TCPOBOP administration (results not shown).

Analysis of cytochrome *P*-450 mRNA levels demonstrated that mRNAs encoding the various cytochrome *P*-450s were also still elevated 3 months after a single injection of TCPOBOP (results not shown). The long-term effects of TCPOBOP were not only reflected in the cytochrome *P*-450 levels but also in liver size. An approximately twofold increase in liver weight was observed 4 days after treatment with TCPOBOP. This increase was still observed 3 months after administration of a single dose of the drug.

In view of the similarities between the abilities of TCPOBOP and dexamethasone to induce cytochrome *P*-450 expression, and particularly the species specificity observed in their inductive effects, we investigated whether TCPOBOP might act through the glucocorticoid receptor. In initial experiments we studied the effect of TCPOBOP on the expression of genes reported to be dexamethasone-inducible and to contain the glucocorticoid-responsive promoter element (GRE). The genes chosen were those encoding MT-II (Hu and Davidson, 1990) and MUPS (Knopf et al., 1983) (Figure 5). These blots were carried out on the same samples as used in the cytochrome *P*-450 analysis. In the short-term experiments, TCPOBOP induced MT-II and MUPS mRNA levels in females and MUPS gene expression in both males and females. Interestingly, phenobarbital also induced MT-II mRNA to a greater extent in female tissue. In the long-term experiments, although the sex differences in induction were less marked, the levels of both MUPS and MT-II mRNA expression remained induced 3 months after TCPOBOP administration, which confirms the prolonged effects of this compound on hepatic gene expression (Figure 5b).

The above data indicated that TCPOBOP may act through the glucocorticoid receptor. In order to obtain more direct evidence that this was the case, the murine mammary tumour virus long terminal repeat, which contains a GRE (Tanaka et al., 1991), was linked to the CAT reporter gene and transfected into ZR-75 cells. CAT activity was determined after exposure to dexamethasone, phenobarbital and TCPOBOP (Table 2). Whereas CAT activity was highly dexamethasone inducible, phenobarbital and TCPOBOP were without effect. In addition, TCPOBOP did not antagonize the dexamethasone-mediated inducibility of CAT activity (results not shown). In further experiments, the C3H10T1/2 cell line, which has constitutive cytochrome *P*-450 expression (Pottenger and Jefcoate, 1990) and is responsive to TCPOBOP (G. Smith and C. R. Wolf, unpublished work), was transfected with a construct comprising the GRE linked to CAT. Interestingly, very low levels of TCPOBOP-inducible CAT activity were detected (results not shown).

The effects of TCPOBOP could be due to direct interaction with a 'receptor-like' protein in hepatocytes or, alternatively, to indirect effects on other tissues such as the pituitary. The pituitary has been shown to play a major role in the regulation of certain cytochrome *P*-450 genes (Skett, 1987; Zaphiropoulos et al., 1989). In order to investigate the possible involvement of this tissue in the effects reported here, TCPOBOP, dexamethasone and phenobarbital were administered to hypophysectomized animals (Figure 6). This procedure had no effect on the ability of these compounds to induce cytochrome *P*-450 levels. Dexamethasone was found to be a potent inducer of both Cyp2c and Cyp3a proteins in these experiments. It is interesting to note that induction by phenobarbital and TCPOBOP was significantly higher in hypophysectomized animals for all the cytochrome *P*-450s studied. This is in agreement with the reported increase in

Figure 7 TCPOBOP induction of Cyp2b protein in primary hepatocyte culture

A primary monolayer culture of hepatocytes was established by collagenase perfusion of the livers of Alderley Park Swiss mice as described in the Materials and methods section. The cells were then treated with either 2 mM phenobarbital (PB), TCPOBOP (2, 10 or 50 μ M) or vehicle alone (0.9% NaCl, 0.25% dimethylformamide). Cell sonicates were run in duplicate on SDS/PAGE using a 9% separating gel, and Cyp2b isoenzyme content was determined by Western-blot analysis. IIB1 and IIB2 are rat CYP2B1 and CYP2B2 standards.

mRNA levels after hypophysectomy (Meehan et al., 1988). Further evidence that these compounds act predominantly by direct effects on hepatocytes was obtained by demonstrating that TCPOBOP could induce Cyp2b expression in a primary culture of mouse hepatocytes (Figure 7).

DISCUSSION

TCPOBOP is an extremely powerful modulator of hepatic gene expression in the mouse. It is also a very potent inducer of liver growth. These data suggest that this compound acts through a hormone-like receptor which modulates liver growth. The existence of such a cellular receptor, and whether it directly modulates both the changes in gene expression and liver growth, is open to speculation.

In agreement with previous studies based on enzyme activity measurements (Poland et al., 1980), the effects of TCPOBOP were found to be very similar to those observed with phenobarbital. Indeed, analysis of cytochrome *P*-450 isoenzyme levels indicated that the inductive effects of these compounds are very similar. TCPOBOP modulates the expression of cytochrome *P*-450 isoenzymes from many distinct gene families as well as the level of expression of cytochrome *P*-450 reductase, epoxide hydrolase and the glutathione *S*-transferase proteins (results not shown). In addition, TCPOBOP induces the expression of other hepatic genes such as those of MUPS and MT-II. On this basis, the compound causes one of the most pleiotropic responses to any foreign chemical known to date.

These effects appear to be predominantly at the transcriptional level, and in most cases a close correlation between expressed protein and mRNA levels was observed.

The most striking property of TCPOBOP is its potency, where a single dose of only 75 μ g to a mouse caused the inductive effects shown. Preliminary studies with 25 μ g indicated that it is equally potent at this dose. This supports the proposal that the effects of the compound are mediated by an intracellular receptor, as it is difficult to conceive that such a low dose of a compound can cause metabolic effects which give rise to such profound and pleiotropic changes in gene expression.

The similarities between TCPOBOP and dexamethasone, particularly their species specificity in cytochrome *P*-450 regulation (Poland et al., 1980; Romano et al., 1986), led us to investigate whether TCPOBOP acts through the glucocorticoid receptor. Interestingly, TCPOBOP induced the expression of several genes which are known to be glucocorticoid-responsive and to contain the GRE. However, TCPOBOP did not activate the GRE directly to any significant extent, and did not compete with dexamethasone for receptor binding. This lack of competition could be explained if TCPOBOP requires metabolism *in vivo* to an active product before exerting its inductive effects. However,

there is no evidence for TCPOBOP metabolism and this therefore appears unlikely.

The above data, together with the requirement for much higher doses of dexamethasone than TCPOBOP for cytochrome *P*-450 induction, makes it likely that TCPOBOP does not act through the glucocorticoid receptor. It could, however, act through an analogous steroid-hormone-like receptor with a lower affinity for dexamethasone. Alternatively, TCPOBOP and dexamethasone may induce cytochrome *P*-450 levels by different mechanisms. It will be interesting to establish the mechanism by which both MUPS and MT-II gene expression is regulated by this compound.

The mechanism of regulation of cytochrome *P*-450 genes by phenobarbital remains unclear. However, recent progress has been made in the identification of a promoter element which is conserved between a *Bacillus megaterium* cytochrome *P*-450 gene and the rat cytochrome *P*-450 CYP2B1 gene (He and Fulco, 1991). The similarities between TCPOBOP and phenobarbital indicate that this promoter may also be involved in gene regulation by TCPOBOP. It would be difficult, however, to explain the species difference in cytochrome *P*-450 regulation by TCPOBOP if this was the only regulatory element involved. The presence of this element in the Cyp2b gene would also not explain the pleiotropic effects of this compound. In this regard, it is interesting that TCPOBOP administration to rats did not induce cytochrome *P*-450s from the 1A or 2B gene families (G. Smith, C. J. Henderson, M. G. Parker, R. White, R. G. Bars and C. R. Wolf, unpublished work). It does, however, cause a slight increase in liver growth.

TCPOBOP could exert its effects on hepatic gene expression either directly or indirectly, for example via the pituitary. Dexamethasone has previously been reported to affect pituitary function (Skett, 1987). Hypophysectomy, however, did not prevent TCPOBOP, phenobarbital or dexamethasone from inducing hepatic cytochrome *P*-450s, indicating that the observed effects of these compounds occur by direct action on hepatocytes. This was confirmed by the finding that TCPOBOP and phenobarbital can induce cytochrome *P*-450 expression in a primary culture of mouse hepatocytes.

In the mouse, TCPOBOP is not metabolized by the cytochrome *P*-450 system (Poland et al., 1980), but accumulates in fat tissue, explaining the longevity of its inductive effects. This also implies that the circulating concentration of the compound is much lower than the injected dose and that the reported potency is an underestimation. We are currently investigating how long these effects on hepatic gene expression are maintained. To date, we have studied up to 6 months after a single injection of TCPOBOP, at which time the changes in cytochrome *P*-450 expression and increase in liver weight are still observed. These data lead to the intriguing possibility that the administration of a single dose of a foreign compound could have profound toxicological consequences months or years after exposure.

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