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Regulatory Roles of AhR

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The abbreviations used are as follows: AhR, arylhydrocarbon receptor; TCDD, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin; Arnt, AhR nuclear translocator; PAS, Per-Arnt-Sim homology; HSP90, heat shock protein 90 kD; XAP2, hepatitis B virus X-associated protein; CYP, cytochrome P-450; XRE, xenobiotic responsive element; RIP140, receptor-interacting protein; 3-MC, 3-methylchoranthrene; LD_{50}, 50% lethal dose.

1. Introduction

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds induce a broad spectrum of biochemical and toxicological effects, such as induction of drug-metabolizing enzymes, teratogenesis, immunosuppression due to thymic involution, and tumor promotion. AhR is a ligand-activated transcription factor that binds to TCDD with an extremely high affinity, and the liganded AhR translocates to nuclei where it forms a heterodimer with Arnt (AhR nuclear translocator) and binds to the XRE (xenobiotic responsive element) sequence in the promoter region of the target genes to activate their expression. Extensive studies of AhR have revealed that most, if not all, of the toxic effects of TCDD are due to AhR. Here, we briefly summarize recent studies on AhR. Because of limited space, we apologize that this review is not comprehensive.

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2. Molecular Anatomy of AhR

AhR and its partner molecule, Arnt, are members of a structurally related gene family with a characteristic structural motif designated as bHLH-PAS (Fig. 1).[1,2] In the N-terminal region, these proteins contain a basic helix-loop-helix (bHLH) motif, which is shared by other transcription factors such as Myc and MyoD, and are involved in DNA binding and hetero- or homodimerization. A sequence consisting of about 250 amino acids adjacent to the C-terminus of the bHLH region corresponds to the PAS (Per, Arnt, Sim) domain, which was initially identified to be a conserved motif between *Drosophila* PER, human ARNT, and *Drosophila* SIM. The PAS domain includes two imperfect repetitions of 50 amino acids, PASs A and B, and is considered to function as an interactive surface for hetero- or homodimer formation. The PAS domain is distributed in a wide variety of proteins involved in circadian rhythm (PER, CLOCK, BMAL1), hypoxia response (HIF-1α, HIF-2α/HLF), neurogenesis (SIM), and transcription coactivation (SRC-1, TIF2) in the animal kingdom and is also found in bacterial proteins functioning as light and oxygen sensors.[3,4]

The ligand-binding domain of AhR is located in the sequence of 230-431 a. a. harboring the PAS B region, and this region overlaps the binding site for Hsp90 making it structurally competent to bind a ligand.[5] In addition, hsp90 bound to PAS B is also known to interact with the bHLH region to mask the nuclear localization signal (NLS) therein, resulting in the cytoplasmic localization of AhR.[6,7] When bound to a ligand, AhR most probably change the mode of binding to Hsp90 to expose the NLS, leading to nuclear translocation of AhR. A recent report has suggested that the Rb pocket sequence, LXCXE, in the C-terminal region of PAS B binds to Rb, thereby affecting the cell cycle in hepatoma cell lines.[8] The transactivation activity is distributed broadly in the C-terminal half of the AhR molecule, while that of Arnt is localized at the C-terminal 43-amino-acid sequence.[9]

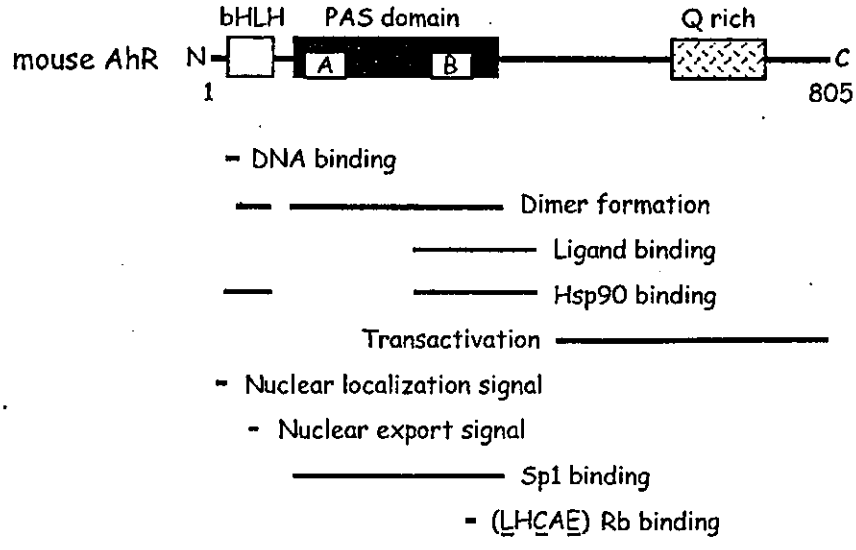

Fig. 1. Map of the functional domains of AhR bHLH: basic helix-loop-helix; PAS: Per/Arnt/Sim homology, Q rich: glutamine rich.

These transactivation activities are transmitted to GTFs (general transcription factors) through interaction with CBP/p300 for Arnt and with RIP140 for AhR as coactivator.[10,11] Arnt contains a constitutive NLS in the N-terminal region of the bHLH domain.[12] In addition, AhR contains a nuclear export signal (NES) in the second helix of the bHLH domain that mediates the nuclear export of the AhR protein followed by proteasome degradation.[13]

3. AhR Homologs

cDNAs of AhR have been cloned from various species of animals ranging from mammals to flies and nematodes (Fig. 2).[2] Karchner *et al.* have cloned cDNAs of two AhR homologs from *Fundulus* (FhAhR1 and FhAhR2),[14] whereas only one AhR homolog has been identified in mammalian species despite extensive efforts. These two homologs of fish AhR mRNAs are derived from their independent genes. Rainbow trout also expresses at least two isoforms of AhR (rtAhR2α and rtAhR2β). Interestingly, the expressions of rtAhR2α and rtAhR2β mRNAs are induced by TCDD, indicating that positive autoregulation may be functional in fish.[15] For invertebrate species, structurally conserved AhR and Arnt homologs have been found in both *Drosophila* and *Caenorhabditis elegans*.[16,17] An AhR homolog of *Drosophila* is Spineless. Deletion of the spineless gene caused morphological transformation in distal legs and antennae of adult flies. Spineless can form a heterodimer with Tango (an Arnt homolog of *Drosophila*), which recognizes the XRE sequence. AHR-1 is the *C. elegans* homolog of AhR and forms a heterodimer with an Arnt homolog of *C. elegans*, AHA-1, which binds the XRE. However, it remains unknown whether or not these invertebrate AhR homologs can mediate the toxicological effects of xenobiotics including dioxin, because nematode AHR-1 appears to exhibit no ability for binding to β-naphthoflavone like the mammalian AhR.[17]

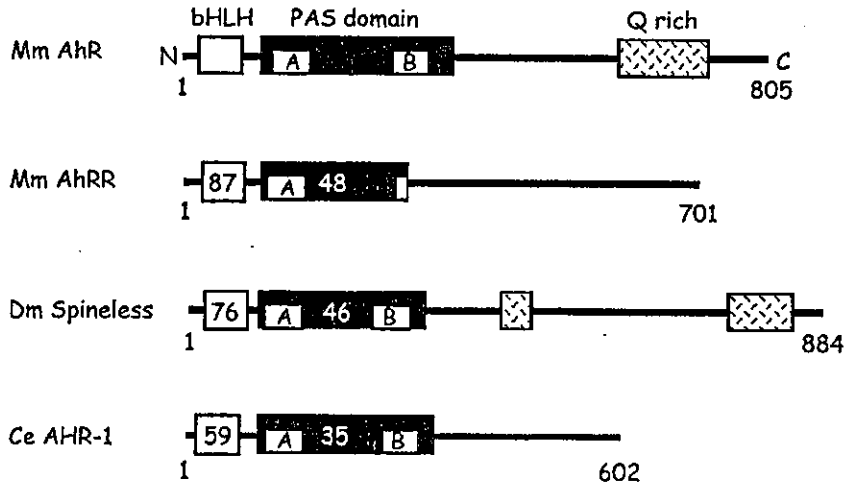

Fig. 2. Amino acid sequence comparison of mouse AhR (Mm AhR), mouse AhRR (Mm AhRR), fruit fly Spineless (Dm spineless) and nematode AHR-1 (Ce AHR-1). Figures indicate the percent amino acid identity with mouse AhR.

4. Mechanisms of Transcriptional Activation by AhR

In the absence of a ligand, AhR exists in the cytosolic complex with Hsp90, co-chaperone p23 and immunophilin-like protein XAP2 (also AIP or ARA9) (Fig. 3).[18,19] p23 and XAP2 are required for maintaining the stability of the Hsp90 complex. Ligand binding to AhR triggers nuclear translocation of AhR, and in nuclei, liganded AhR replaces its partner molecule with Arnt, resulting in binding to its recognition sequence, XRE (also named DRE or AhRE) in the promoter of the genes for a series of drug metabolizing enzymes and other TCDD-inducible genes. Binding of the AhR/Arnt heterodimer to XREs remodels the chromatin structure and facilitates the association of other transcription factors, including Sp1.[10,20] As described above, the transactivation activity of the AhR/Arnt is mediated through CBP/p300 and RIP140.

Phosphorylation of the AhR/Arnt heterodimer is reported to also be important for transactivation, because the binding activity of the AhR/Arnt heterodimer to the XRE sequence is abolished by the phosphatase treatment.[21] Although the phosphorylation sites of AhR and Arnt were determined to be tyrosine 372 and serine 348, respectively, replacements of these amino acids with alanine had no effect on the DNA binding activity of the heterodimer, indicating that other phosphorylation sites may be involved in the DNA binding activity.[21,22]

It has been reported that liganded AhR is rapidly degraded via the ubiquitin/proteasome

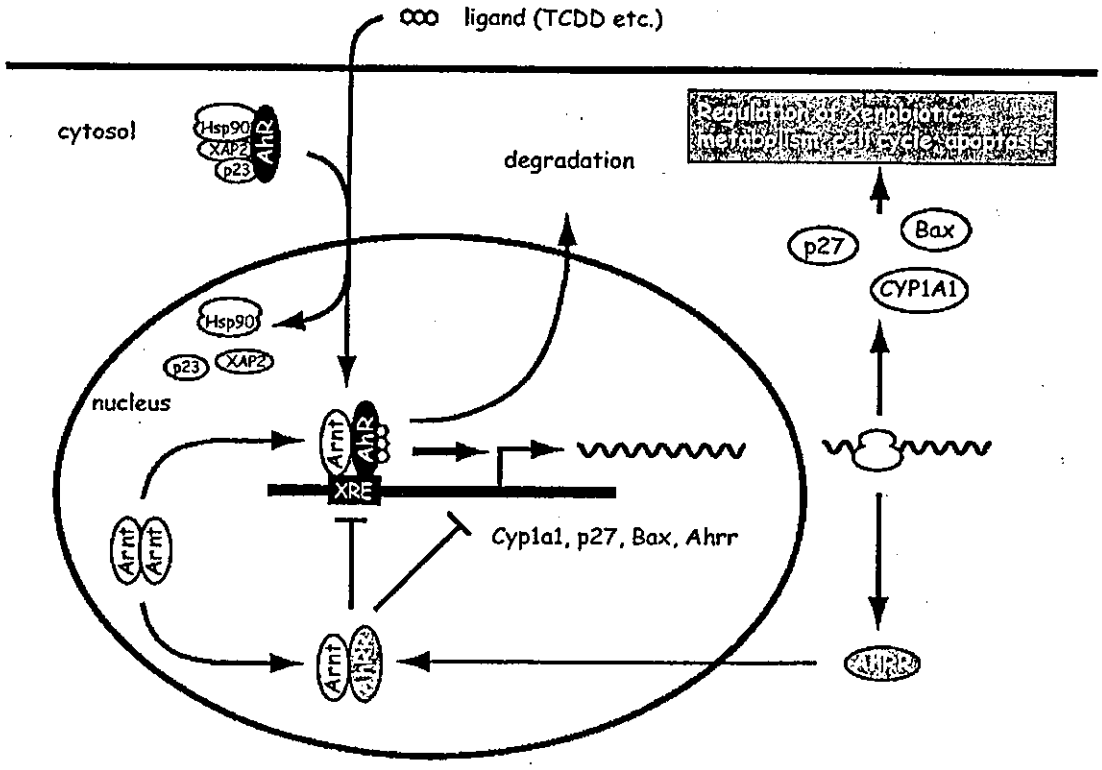

Fig. 3. AhR signaling pathway and negative feedback regulation of AhR function by AhRR.

pathway after transactivation of the target genes.[23,24] Nuclear localization and phosphorylation of AhR were necessary for degradation. Interestingly, an inhibitor of protein synthesis, cycloheximide, inhibited the ligand-dependent degradation of AhR,[25] although its precise mechanism remains unknown.

5. Target Genes of AhR

A number of phase I (CYP1A1, 1A2, 1B1) and phase II (NADP(H):oxidoreductase, GST-Ya, UDP-glucronosyltransferase) drug metabolizing enzymes (DMEs) are known to be induced by AhR ligands. In addition to these enzymes, AhR ligands upregulate genes involved in cell proliferation (TGF-β, IL-1β and PAI-2), cell cycle regulation ($p27^{KLP1}$ and jun-B), apoptosis (Bax), and so on.[1,26,27] Most of these genes are found to contain the XRE sequence in their regulatory region, indicating that ligand-activated AhR upregulates the expression of these genes by directly binding to the XRE sequences (i.e. primary effects). On the other hand, indirect mechanisms (i.e. secondary effects) of the induction of multidrug resistance gene *mdr1* by 3-MC were also reported.[28] In this case, 3-MC induces DMEs in an AhR-dependent manner and the induced DMEs metabolically activate 3-MC to genotoxic metabolites that modify nucleotide bases of DNA. The modified DNA stabilizes p53, and the accumulated p53, in turn, upregulates the expression of MDR1 (multidrug resistance 1) by binding to its cognate DNA sequence in the promoter. In HepG2 cells, it was reported in the DNA microarray analysis that 310 genes are either up- or downregulated by the TCDD treatment.[29] Of the 310 genes affected by TCDD, 108 genes are still observed to be affected even by the simultaneous treatment with cycloheximide, while the altered expression of the remaining 202 genes by the TCDD treatment becomes unchanged by the treatment with the protein synthesis inhibitor. These results suggested that the expression of the former 108 genes is directly regulated by TCDD without protein synthesis, while the regulation of the remaining 202 genes by TCDD requires protein synthesis.

Safe *et al.* reported that the enhanced expression of cathepsin D and c-fos with estrogen treatment was inhibited in the presence of TCDD through the XRE sequence.[30] Although precise mechanisms of this inhibition still remain controversial, there is some possibility that AhR may play an inhibitory role in the transcription by binding to a specific sequence of XRE, designated as inhibitory XRE.

6. Modulation of AhR Function

In addition to Arnt, a number of proteins are reported to interact with AhR. As described above, AhR exists in the cytoplasm in the complex of Hsp90, p23 and XAP2 in the absence of ligand. It is also reported that the pp60 src protein is found in this AhR-chaperone complex.[31] As soon as AhR binds a ligand, src kinase is released from the AhR-hsp90 complex and activates Ras-dependent signaling cascades.[32] At the initiation step of transcription, the AhR/Arnt heterodimer and Sp1 synergistically bind to their respective cognate DNA sequences by interacting with each other, and the bound AhR/Arnt heterodimer transmits the transactivation activity to GTFs by recruiting CBP/p300

and RIP140.[10] Recent studies have demonstrated that Rb and NF-κB also interact with AhR. Liganded AhR stabilizes the complex of Rb and E2F by binding to Rb, thereby enhancing the repression of the transcriptional activity of E2F and, therefore, the cell cycle is arrested.[33] It is also suggested that Rb functions as a co-activator of AhR by binding with AhR.[34] The ligand-activated AhR and the RelA subunit of NF-κB interact with each other and mutually inhibit each other from binding to each of their respective recognition sequences, when their cognate sequences lie on the separate gene promoter.[35] On the other hand, TCDD and TPA synergistically induce the expression of the AhRR gene driven by the XRE and NF-κB binding sequences in the promoter, probably through the interaction between AhR and NF-κB.

AhRR has a bHLH-PAS sequence with a high similarity to that of AhR and inhibits the transcription activity of AhR by competing with AhR for binding to Arnt and the XRE sequence (Figs. 2 and 3).[36] Since AhRR expression is induced by TCDD through AhR/Arnt heterodimer, AhR and AhRR form a regulatory feedback loop in the xenobiotic signal transduction.

A hypoxia signal also inhibits AhR-dependent transactivation.[37] In hypoxia, HIF-1α is stabilized and translocates to the nucleus where it heterodimerizes with Arnt in competition with AhR. Thus, xenobiotic and hypoxia signal transduction pathways interfere with each other by competing for a common partner molecule Arnt.

7. Polymorphism of AhR

It is well known that there is a marked strain and species difference in sensitivity to TCDD[1,2]. LD_{50} values of TCDD vary over a 5000-fold range among different species. For example, LD_{50} values vary from 1 μg/kg for the guinea pig, the most sensitive animal, to > 5000 μg/kg for the hamster, the most resistant. In mice, the difference in the responsiveness to TCDD among strains depends on AhR alleles. cDNA cloning of responder (C57BL) and nonresponder (DBA) mice revealed that Ala-to-Val substitution at codon 375 and the C-terminal extension in DBA mice ablate the binding affinity of AhR toward TCDD.[38] Polymorphism in the AhR loci is also found in the rat.[39] TCDD-sensitive Long-Evans (L-E) (LD_{50}, 10–20 μg/kg) and insensitive Han/Wistar (H/W) (LD_{50}, > 9600 μg/kg) rats show about 1000-fold difference in sensitivity. cDNA cloning indicates that L-E rat AhR cDNA sequence is the same as that of Sprague-Dawley rat previously determined.[39] In contrast, H/W rat AhR cDNA was revealed to carry a point mutation in the first base of intron 10, resulting in a different sequence of the C-terminal transactivation domain. In humans, one genetic variation has been reported at codon 554, resulting in amino acid change from Arg to Lys.[40,41] However, this mutation does not seem to be related to any phenotype of TCDD toxicity in humans.

8. AhR KO Mice

Three groups reported the generation of AhR-knockout mice using targeted disruption of the Ahr locus.[42–44] AhR-null mice were born in normal Mendelian genetics, but their growth was retarded for the first 3 weeks of life. AhR-null mice were revealed to be

defective in hepatic development, immune system development, and retinoic acid metabolism. It is reported that female AhR-null mice had difficulty in maintaining conception, lactation, and rearing of pups to weaning.[45] Recently, AhR deficiency has been revealed to cause abnormal hepatic vascular structures.[46] Concerning xenobiotic metabolism, the lack of AhR abolished the inducible expression of CYP1A1 and 1A2 in mice in response to polycyclic aromatic hydrocarbons and resulted in a loss of susceptibility to chemical carcinogenesis by benzo[a]pyrene and teratogenesis caused by TCDD.[44,47] Although the genes responsible for TCDD-induced teratogenesis remain to be identified, the loss of the inducible expression of CYP1A1, 1A2 and 1B1 which metabolically activate various procarcinogens, is considered to be a cause of resistance to chemical carcinogenesis. In addition, AhR together with Arnt has been shown to upregulate the expression of DNA polymerase κ which duplicates DNA in a error-prone manner.[48] This is a valid reason why AhR is also involved in the promotion of chemical carcinogens.

9. AhR Ligand

It is well known that TCDD is the most potent ligand of AhR with an extremely low Kd value. Other types of halogenated aromatic hydrocarbons such as PCB and polycyclic aromatic hydrocarbons such as benzo[a]pyrene also function as ligand to AhR. Some flavonoids function as antagonists at low concentrations (~0.5 μM), while they function as an agonist for AhR at higher concentrations (>10 μM).[49,50] It has been reported that 7-ketocholesterol functions as an AhR antagonist and actually antagonizes the TCDD effects *in vivo*.[51] This antagonistic effect of 7-ketocholesterol has been suggested to have physiological significance, because it was observed at its physiological plasma concentration. Resveratrol, a plant steroid, was also reported to be an antagonist for AhR.[52] For some time it has been an interesting problem to find a true and intrinsic ligand of AhR. Recently, a number of endogenous AhR ligands have been reported to have a high affinity toward AhR. Most of these chemicals are tryptophan derivatives. UV-irradiated or ozone-treated tryptophan products function as an AhR agonist.[53] Some of the purified products were reported to function as a potent ligand of AhR. Indirubin and indigo isolated from urine were reported to function as an even more potent ligand of AhR than TCDD in a yeast assay system.[54] However, TCDD was reported to be a poor inducer of the AhR-dependent reporter gene in yeast, probably due to its permeability.[55] It will be difficult to identify true intrinsic ligands of AhR, until the intrinsic roles of AhR, other than gene regulation of a series of drug-metabolizing enzymes, are clarified.

10. Conclusion

In summary, AhR has originally identified as a mediator of dioxin toxicities. However, recent studies have suggested that AhR plays a regulatory role in homeostasis and development, and its detailed mechanisms remain to be clarified in future studies.

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Metabolite of 2,2′,4′,5-tetrabromobiphenyl, 3-methylsulphonyl-2,2′,4′,5-tetrabromobiphenyl, a potent inducer of CYP2B1/2 in rat

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1. 3-Methylsulphonyl- and 4-methylsulphonyl-2,2′,4′,5-tetrabromobiphenyls (3-$MeSO_2$- and 4-$MeSO_2$-TetraBrBs) were detected in the liver, lung, kidney, adipose tissue and faeces of the 2,2′,4′,5-tetrabromobiphenyl (TetraBrB)-dosed rat.

2. The administration of 0.05–2.0 μmol kg^{-1} doses of 3-$MeSO_2$-TetraBrB produced corresponding increases in the hepatic concentration of the methyl sulphone metabolite, corresponding increases in the content of total cytochrome P450, and corresponding increases in the activities of 7-benzyloxy-, 7-ethoxy- and 7-pentoxyresorufin *O*-dealkylases. The inducing effects of the 3-$MeSO_2$-TetraBrB (0.2 μmol kg^{-1}), both on the content of total P450 and on the activities of the three alkoxyresorufin *O*-dealkylases, were higher than that of the parent TetraBrB (342 μmol kg^{-1}).

3. The major phenobarbital (PB)-inducible forms of P450, CYP2B1, CYP2B2, CYP3A2 and CYP2C6, were substantially induced by 3-$MeSO_2$-TetraBrB, but CYP1A1 and CYP1A2 were not. On the other hand, the activities of drug-metabolizing enzymes and the four PB-inducible forms of P450 were unchanged by 4-$MeSO_2$-TetraBrB treatment.

4. The induction profiles of these enzymes and P450 forms in rat treated with 3-$MeSO_2$-TetraBrB were similar to those treated with PB.

5. The inducing ability of 3-$MeSO_2$-TetraBrB (0.5 μmol kg^{-1}) both on the activities of the three alkoxyresorufin *O*-dealkylases and on the contents of four PB-inducible forms of P450 was roughly equal to that of PB (431 μmol kg^{-1} twice at a 24-h interval) or 3-$MeSO_2$-2,2′,4′,5-tetrachlorobiphenyl (1 μmol kg^{-1}). It is noteworthy that the effects of 3-$MeSO_2$-TetraBrB on the drug-metabolizing enzymes CYP2B1 and CYP2B2 were several thousand-fold higher than those of parent TetraBrB, while the effect of its isomeric 4-$MeSO_2$-TetraBrB were not.

6. The extent of hepatic accumulation of the 3-$MeSO_2$ metabolite after the administration of TetraBrB (342 μmol kg^{-1}) was almost the same as that after the administration of 3-$MeSO_2$-TetraBrB (0.1–0.2 μmol kg^{-1}). The relationship between the hepatic concentration of the 3-$MeSO_2$ metabolite and the extent of enzyme induction after the administration of TetraBrB or 3-$MeSO_2$-TetraBrB suggests that 3-$MeSO_2$-TetraBrB plays an important role in the induction of microsomal drug-metabolizing enzymes by TetraBrB.

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Introduction

Polybrominated biphenyls (PBrBs) have been used as fire retardants for moulded plastic parts such as the cases of televisions, typewriters and business machines (Carter 1976, WHO 1994). In 1973, for a few months, many Michigan residents unknowingly ate meat and dairy products contaminated with PBrBs (Dunckel 1975, Fries 1988). PBrBs are stable and lipophilic, and tend to accumulate in lipid-rich tissues in rat, cow and marine animals (Fries and Marrow 1975, Matthews *et al.* 1977, De Boer *et al.* 1998). Just like polychlorinated biphenyls (PCBs), PBrBs have been reported to elicit a number of toxic and other biological responses in diverse mammalian species (Garthoff *et al.* 1977, Hass *et al.* 1978, Fries 1988) and there are many studies of the toxicity of PBrBs (Garthoff *et al.* 1977, Dicarlo *et al.* 1978, Hass *et al.* 1978, Fries 1988). However, the biotransformation of PBrB to methylsulphonyl ($MeSO_2$) metabolite(s) in animals has not been reported to date. If PBrBs are biotransformed to $MeSO_2$ metabolite(s) in animals, $MeSO_2$-PBrBs may have biological activities and/or toxicological effects similar to the $MeSO_2$-PCBs.

We reported that the 3-$MeSO_2$ metabolites of PCBs are inducers of rat hepatic microsomal drug-metabolizing enzymes CYP2B1 and CYP2B2 (Kato *et al.* 1995a, b, 1997), and their inducing potency are several hundred times higher than the parent PCBs (Kato *et al.* 1995a, 1997). We also showed that the seven 3-$MeSO_2$ and two 4-$MeSO_2$ metabolites of tetra-, penta- and hexachlorobiphenyls reduced serum thyroxine (T_4) levels (Kato *et al.* 1998a, 1999), and the seven 3-$MeSO_2$-PCBs and 4-$MeSO_2$-2,2′,4′,5,5′-pentachlorobiphenyl increases in hepatic T_4 glucuronidation after the administration of the methyl sulphones possibly because of the induction of both UGT1A1 and UGT1A6 caused the reduction of serum T_4 levels (Kato *et al.* 2000).

The current study attempts to identify the $MeSO_2$ metabolites in the liver of rat after the administration of a PBrB congener, 2,2′,4′,5-tetrabromobiphenyl (TetraBrB), and, second, to compare the inductive effects of 3- and 4-$MeSO_2$-TetraBrBs on the drug-metabolizing enzyme system, with the parent TetraBrB.

Materials and methods

Chemicals

TetraBrB was synthesized by using the Cadogan (1962) coupling reactions. 3- and 4-$MeSO_2$-TetraBrBs and 3-$MeSO_2$-2,2′,4′,5-tetrachlorobiphenyl (3-$MeSO_2$-TetraCB) were prepared as described by Haraguchi *et al.* (1987). The purity of these compounds was > 99% as determined by GC. 3-$MeSO_2$-4-methyl-2,3′,4′,5,5′-pentachlorobiphenyl (3-$MeSO_2$-4-Me-2,3′,4′,5,5′-pentaCB) was used as the internal standard. Panacete 810 (a mixture of medium-chain triglycerides) was purchased from Nippon Oils and Fats Co., Ltd (Tokyo, Japan); 7-benzyloxy-, 7-ethoxy- and 7-pentoxyresorufins were from Sigma Chemical Co., Ltd (St Louis, MO, USA); $NADP^+$, NADPH, glucose 6-phosphate disodium salt and glucose 6-phosphate dehydrogenase were from Oriental Yeast Co., Ltd (Osaka, Japan); and phenobarbital (PB) sodium was from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The microsomal cytochrome P450 standards and antibodies against purified P450s were obtained from Daiichi Pure Chemicals Co., Ltd (Tokyo, Japan). A polyclonal antibody raised against rat CYP2B1, which detects both rat CYP2B1 and CYP2B2, a polyclonal antibody raised against rat CYP2C6 and CYP3A2, which detects rat CYP2C6 and CYP3A2, respectively, and a polyclonal antibody raised against rat CYP1A1, which detects both rat CYP1A1 and CYP1A2, were used. Other chemicals were obtained from commercial sources.

The spectroscopic properties of tetraBrB and its metabolites were as follows.

- TetraBrB: mass spectrum; *m/z* (relative abundance, %) 470 (100, M + 4), 468 (74, M + 2), 466 (18, M^+), 387 (33, M^+-Br), 308 (44, M^+-2Br).
- *3-$MeSO_2$-TetraBrB*: retention time on glc; 27.2 min. Mass spectrum; *m/z* (relative abundance, %):

548 (23, $M^+ + 4$), 546 (20, $M^+ + 2$), 544 (6, M^+), 465 (38, M^+-Br), 469 (100, M^+-Br+4), 386 (8, M^+-2Br), 228 (36, M^+-MeSO$_2$-3Br); ^{1}H-NMR δ (ppm): 3.35 (3H, s, SO_2CH_3), 7.10 (1H, d, J = 8.0 Hz, 6′-H), 7.56 (1H, dd, J = 1.8, 8.0 Hz, 5′-H), 7.61 (1H, d, J = 2.2 Hz, 6-H), 7.87 (1H, d, J = 1.8 Hz, 3′-H), 8.40 (1H, d, J = 2.2 Hz, 4-H).

- *4-MeSO$_2$-TetraBrB*: retention time on glc; 28.1 min. Mass spectrum; *m/z* (relative abundance,%) 548 (100, $M^+ + 4$), 546 (75, $M^+ + 2$), 544 (20, M^+), 481 (5, M^+-MeSO), 465 (36, M^+-Br), 386 (22, M^+-2Br), 228 (62, M^+-MeSO$_2$-3Br); ^{1}H-NMR δ (ppm) 3.29 (3H, s, SO_2CH_3), 7.08 (1H, d, J = 8.1 Hz, 6′-H), 7.55 (1H, dd, J = 1.8, 8.1 Hz, 5′-H), 7.62 (1H, d, J = 2.2 Hz, 6-H), 7.88 (1H, d, J = 1.8 Hz, 3′-H), 8.43 (1H, d, J = 2.2 Hz, 3-H).

Animal treatment

Male Wistar rats, weighing 180–200 g, were housed three or four per cage with free access to food (MF, Oriental Yeast Co.) and tap water, and maintained on a 12-h dark/light cycle (08:00–20:00 hours light) in a room at a controlled temperature (24.5 ± 1°C) and humidity (55 ± 5%). Rats received an i.p. injection of TetraBrB (342 μmol kg^{-1}) and its MeSO$_2$ derivatives (0.05–2.0 μmol kg^{-1}) dissolved in Panacete 810 (5 ml kg^{-1}). Control animals received an equivalent volume of the vehicle. All rats were starved for 18 h before killing by decapitation at the designated time after the dosing.

Microsomal preparation and enzyme assays

Microsomes were prepared according to the procedure of Kato *et al.* (1995b). The protein content was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Total P450 content was estimated according to the method of Omura and Sato (1964). The activity of alkoxyresorufin *O*-dealkylase in microsomes was determined by the method of Burke *et al.* (1985). The immunoblotting and immunochemical quantitation were performed as described by Imaoka *et al.* (1987).

Isolation of metabolites

Sample cleanup and quantification were carried out according to our previous methods (Haraguchi *et al.* 1997). Briefly, liver samples were homogenized with acetone/*n*-hexane (2:1 v/v). An internal standard (3-MeSO$_2$-4-Me-2,3′,4′,5,5′-pentaCB, 70 ng) was added to each extract and the mixtures subjected to a gel permeation column packed with Bio-Beads S-X3 (50 g, Bio-Rad Laboratories, Hercules, CA, USA). Dichloromethane/*n*-hexane (1:1) was used as a mobile phase at a flow rate of 4 ml min^{-1}. The metabolite fraction (120–200 ml) was collected and subjected to GC analysis. Other tissue samples were purified as described for the liver.

Analytical methods

^{1}H-NMR spectra were obtained on a JEOL GSX-500 spectrometer (500 MHz). The samples were dissolved in chloroform-*d* with TMS as an internal standard. GC/MS was carried out on a JMS-AX505W (JEOL) apparatus connected to a JMA-DA5000 data system in the EI mode. The GC instrument was fitted with a DB-5MS fused silica capillary column (30 m × 0.25 mm i.d., J&W Scientific, Inc., Folsom, CA, USA) with helium as a carrier gas. Injection was carried out in the splitless mode. The oven temperature was programmed from 70 (2 min) to 220°C at 20°C min^{-1} and then to 280°C at 4°C min^{-1}. The GC for quantification was performed on a GC-14A (Shimadzu Co., Kyoto, Japan) instrument equipped with a ^{63}Ni electron-capture detector (ECD) with column conditions analogous to those described for GC/MS.

The metabolites in samples were identified on GC/MS by comparison with authentic synthesized metabolites. Recoveries of 3-MeSO$_2$-4-Me-2,3′,4′,5,5′-pentaCB added to liver samples before extraction were > 85%. The quantification of metabolites was performed on a GC/ECD apparatus using 3-MeSO$_2$-4-Me-2,3′,4′,5,5′-pentaCB as an internal standard.

Statistics

The results were statistically analysed according to a Student's *t*-test.

Results

Identification of the MeSO$_2$ metabolites of TetraBrB

Figure 1 shows the GC/MS profiles of liver extracts from the TetraBrB-dosed rat. The mass spectra of metabolites MT-1 and MT-2 in the total ion chromatogram showed the molecular ion peaks (M^+) at *m/z* 544 and other characteristic

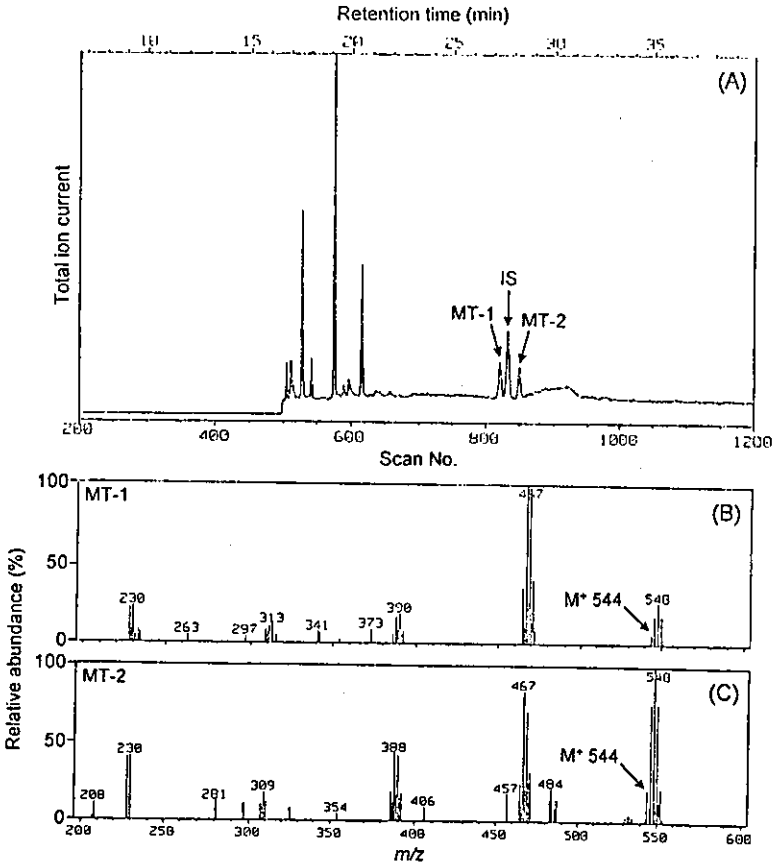

Figure 1. Total ion chromatogram (A) and mass spectra (B, C) of the liver extract from the TetraBrB-administered rat.

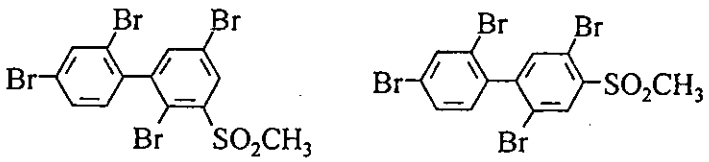

Figure 2. Chemical structures of the methyl sulphone metabolites of TetraBrB.

peaks at m/z 465, [M^+-Br]; at m/z 386, [M^+-2Br], and at m/z 228, [M^+-$MeSO_2$-3Br]. In addition, the mass spectra of MT-2 showed the characteristic peaks at m/z 481, [M^+-MeSO]. Thus, the spectral data suggested the presence of methylsulphone metabolites in the liver. The mass spectra and GCt_R of MT-1 and MT-2 (27.2 and 28.1 min, respectively) corresponded with those of authentic 3- and 4-$MeSO_2$-TetraBrBs, respectively. From these results, MT-1 and MT-2 were identified as 3- and 4-$MeSO_2$-TetraBrBs, respectively (figure 2). Both 3- and 4-$MeSO_2$-TetraBrBs were also detected in the lung, kidney, adipose tissue and faeces (data not shown).

Table 1 shows the concentration of TetraBrB, and 3-$MeSO_2$ and 4-$MeSO_2$ metabolites in the liver, lung, kidney and adipose tissue 96 h after the i.p. administration of 342 μmol kg^{-1} TetraBrB.

Table 1. Tissue concentrations of TetraBrB and its methyl sulphone metabolites after the administration of TetraBrB to rat.

	TetraBrB or methyl sulphone concentration (nmol g^{-1} tissue)		
Tissue	TetraBrB	3-$MeSO_2$-TetraBrB	4-$MeSO_2$-TetraBrB
Liver	4.54 ± 0.70	0.33 ± 0.05	0.28 ± 0.04
Lung	0.82 ± 0.03	0.08 ± 0.02	3.22 ± 0.58
Kidney	1.89 ± 0.30	0.06 ± 0.003	0.41 ± 0.05
Adipose tissue	151.83 ± 36.36	1.39 ± 0.34	1.80 ± 0.45

Rats were given 342 μmol kg^{-1} TetraBrB (i.p.) and killed 96 h after administration. Results are the mean ± SE for three to four animals.

The concentration of TetraBrB was highest (152 nmol g^{-1}) in adipose tissue, and was approximately 1–5 nmol g^{-1} in the liver, lung and kidney. The 3-$MeSO_2$ metabolite accumulated in the order adipose tissue > liver > lung > kidney. The concentration of the 4-$MeSO_2$ metabolite was the highest in the lung followed by adipose tissue, kidney and liver.

Induction of drug-metabolizing enzymes by 3-$MeSO_2$-TetraBrB

The time-course of accumulation of 3-$MeSO_2$-TetraBrB in the liver after the i.p. administration of 0.35 μmol kg^{-1} 3-$MeSO_2$-TetraBrB is shown in figure 3, reaching a maximum at around 168 h.

Figure 4 shows the time-courses of the content of total P450 and the activities of alkoxyresorufin *O*-dealkylases in liver microsomes after the i.p. administration of 0.35 μmol kg^{-1} 3-$MeSO_2$-TetraBrB. The content of total P450 and activities of 7-benzyloxy-, 7-ethoxy- and 7-pentoxyresorufin *O*-dealkylases significantly increased 48 h after dosing, and were 2.0-, 59-, 3.6- and 33-fold of control (0 h) levels, respectively, at 96 h, and remained elevated at 168 h post-dosing.

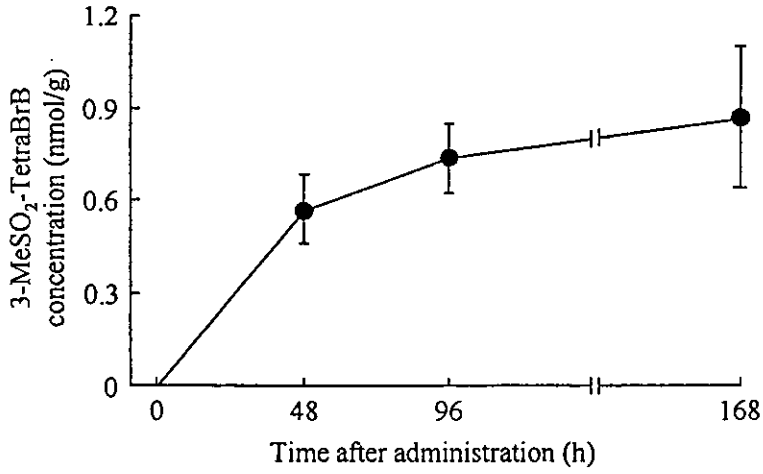

Figure 3. Time-course of the liver concentration of the methyl sulphone after the administration of 3-$MeSO_2$-TetraBrB to rat. Rats were given 0.35 μmol kg^{-1} 3-$MeSO_2$-TetraBrB (i.p.) and killed at the indicated times after administration. Each point represents the mean ± SEM (vertical bars) for four animals.

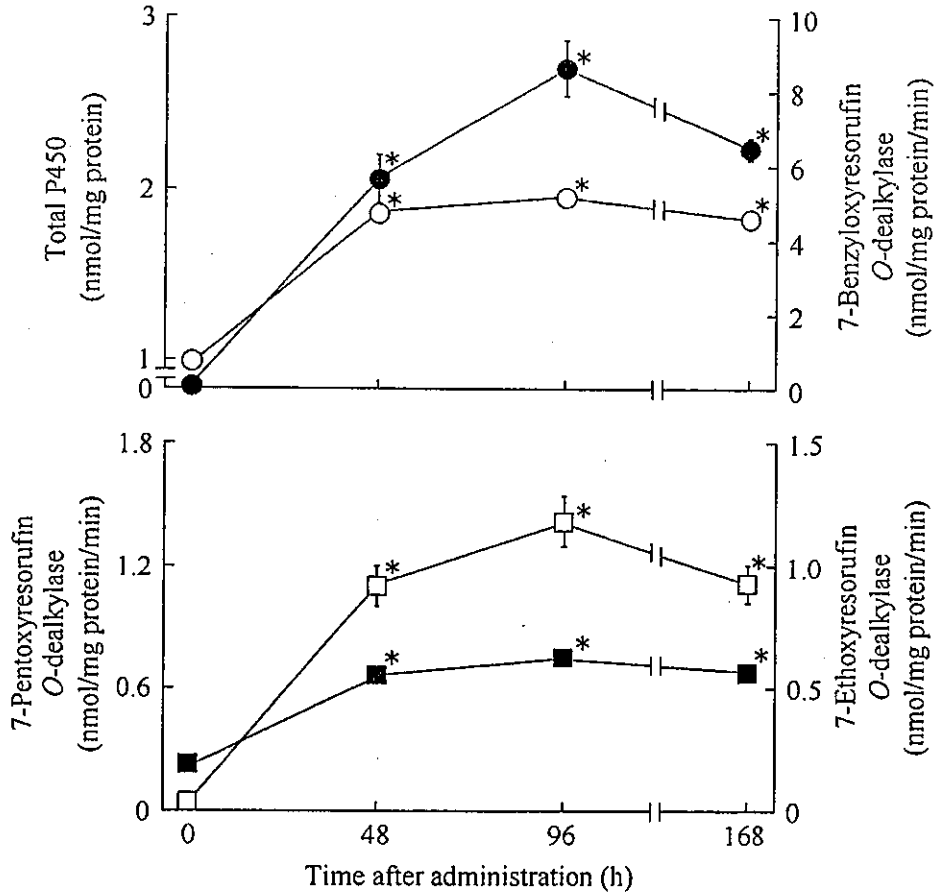

Figure 4. Effects of 3-$MeSO_2$-TetraBrB on the content of total P450 and the activities of drug-metabolizing enzymes of rat liver microsomes. The experimental conditions were the same as described in figure 3. ○, Total P450; ●, 7-benzyloxyresorufin *O*-dealkylase; □, 7-pentoxyresorufin *O*-dealkylase; ■, 7-ethoxyresorufin *O*-dealkylase. Each point represents the mean ± SEM (vertical bars) for five to eight animals. $^*P < 0.001$, significantly different from the control (0 h).

To clarify the relationship between hepatic concentration and the inducing effect on the drug-metabolizing enzymes, we examined the activities of drug-metabolizing enzymes, the contents of P450 forms and the hepatic concentration of methyl sulphone 96 h after the administration of 3-$MeSO_2$-TetraBrB at various doses.

As shown in figure 5, the hepatic concentration of 3-$MeSO_2$-TetraBrB increased in proportion to the dose of 3-$MeSO_2$-TetraBrB. As shown in figure 6, 0.05 μmol kg^{-1} 3-$MeSO_2$-TetraBrB significantly increased both the content of total P450 and activities of 7-benzyloxy-, 7-ethoxy- and 7-pentoxyresorufin *O*-dealkylases. The methyl sulphone caused dose-dependent increases in the content of total P450 and in the extent of dealkylation of 7-benzyloxyresorufin and 7-pentoxyresorufin in the range 0.05–2.0 μmol kg^{-1}, and of 7-ethoxyresorufin of liver microsomes in the range 0.05–0.35 μmol kg^{-1}.

The levels of PB-inducible forms of P450 in hepatic microsomes after the administration of 3-$MeSO_2$-TetraBrB to rat are shown in figure 7. A 0.1 μmol kg^{-1} dose of 3-$MeSO_2$-TetraBrB significantly induced CYP2B1, CYP2B2, CYP3A2 and CYP2C6. The methyl sulphone produced dose-related inductions of CYP2B1, CYP2B2 and CYP3A2 in liver microsomes in the range 0.1–0.5 μmol kg^{-1}. The

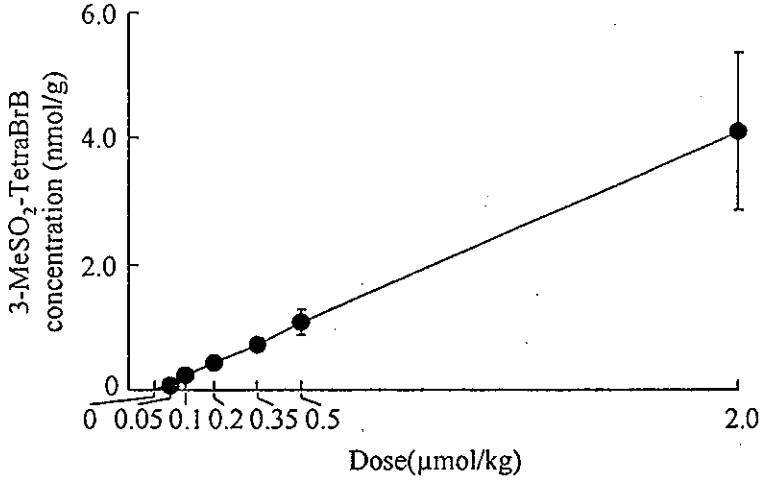

Figure 5. Liver concentrations of 3-MeSO$_2$-TetraBrB after the administration of 3-MeSO$_2$-TetraBrB in graded doses to rat. Rats were given 3-MeSO$_2$-TetraBrB (i.p.) at the various doses indicated and killed 96 h after administration. Each point represents the mean ± SEM (vertical bars) for four animals.

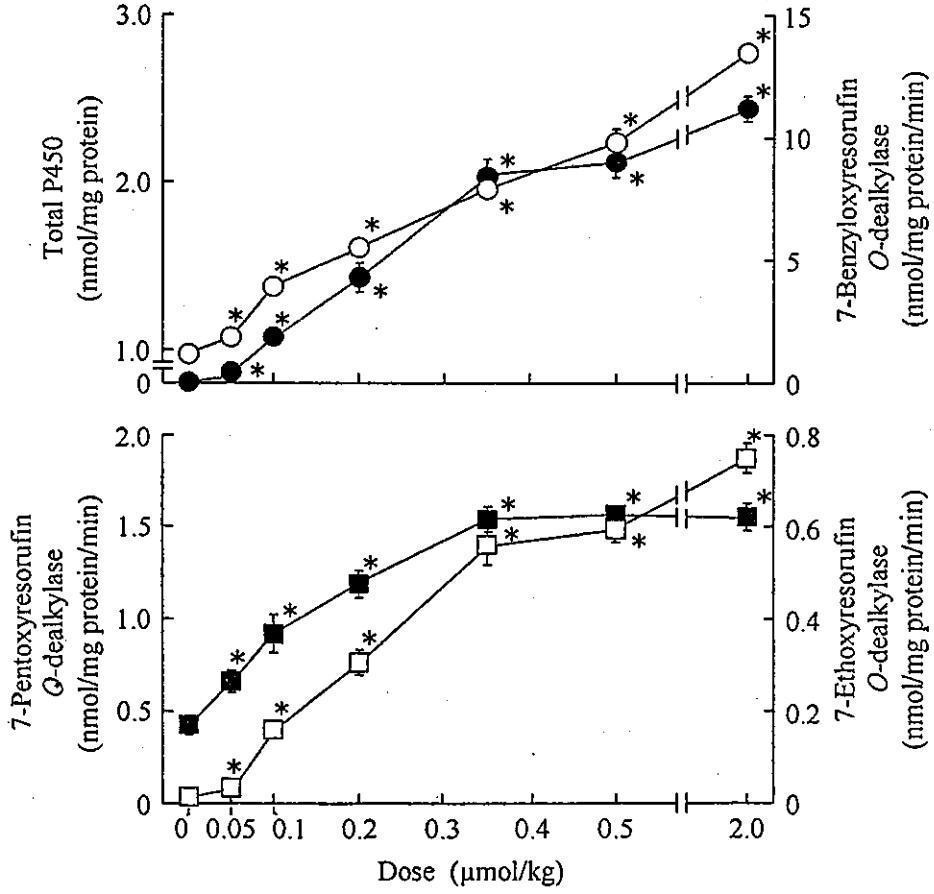

Figure 6. Effects of graded doses of 3-MeSO$_2$-TetraBrB on the content of total P450 and the activities of drug-metabolizing enzymes of liver microsomes in rat. The experimental conditions were the same as described in figure 5. ○, Total P450; ●, 7-benzyloxyresorufin *O*-dealkylase; □, 7-pentoxyresorufin *O*-dealkylase; ■, 7-ethoxyresorufin *O*-dealkylase. Each point represents the mean ± SEM (vertical bars) for five to eight animals. $^*P < 0.05$, significantly different from the control.

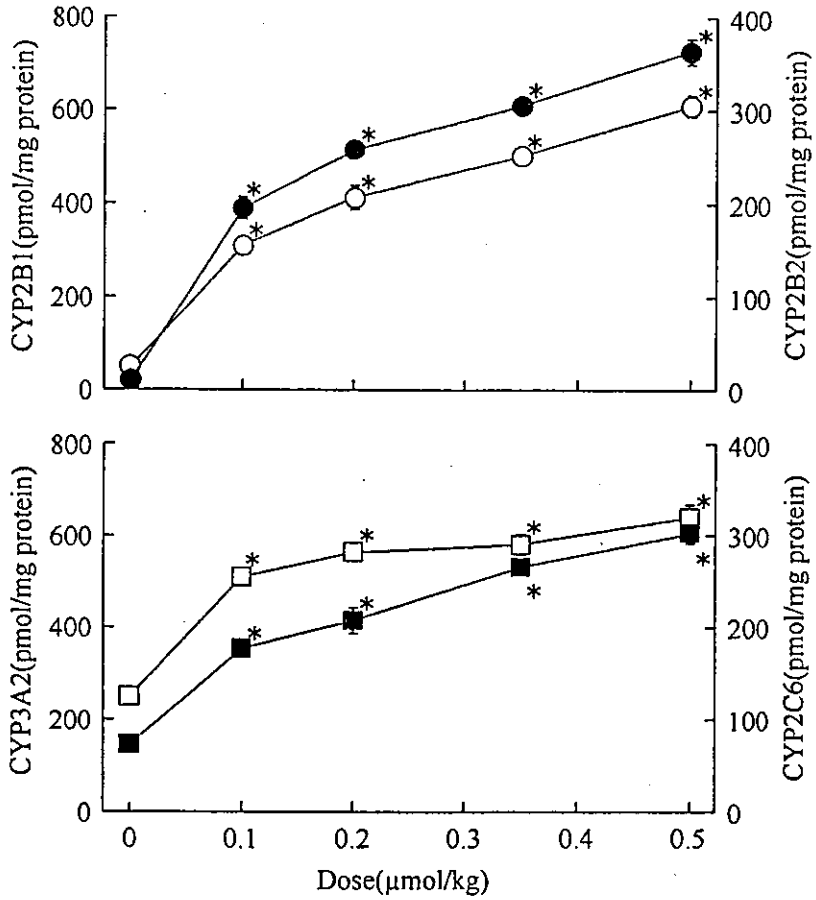

Figure 7. Effects of graded doses of 3-$MeSO_2$-TetraBrB on the contents of P450 forms in rat liver microsomes. The experimental conditions were the same as described in figure 5. The contents of P450 forms were assayed by densitometry of nitrocellulose immunoblotted from an SDS-polyacrylamide gel. Hepatic microsomes (1–5 μg protein) were analysed by Western blotting. Measurement was done with duplicates of three to seven different preparations of microsomes. ●, CYP2B1; ○, CYP2B2; ■, CYP3A2; □, CYP2C6. Each point represents the mean ± SEM (vertical bars) for three to seven animals. *$P < 0.001$, significantly different from the control.

major PB-inducible forms, CYP2B1 and CYP2B2, were induced 35- and 11.4-fold of control by this methyl sulphone at 0.5 μmol kg^{-1}, respectively. In contrast, CYP1A1 was not detected, and no change was observed in CYP1A2 content after the administration of 3-$MeSO_2$-TetraBrB (0.5 μmol kg^{-1}) (data not shown).

Comparative studies of inducing effects

The effect of 3-$MeSO_2$-TetraBrB on the content of total P450, on the activities of drug-metabolizing enzymes and on the contents of P450 forms were compared with that of 3-$MeSO_2$-TetraCB and PB (figures 8 and 9).

The inducing abilities of 0.5 μmol kg^{-1} 3-$MeSO_2$-TetraBrB both on the content of total P450 and activities of the three alkoxyresorufin *O*-dealkylases were almost the same as that of 1 μmol kg^{-1} 3-$MeSO_2$-TetraCB or that of 431 μmol kg^{-1} PB twice at a 24-h interval (figure 8). A similar profile was observed for the induction of CYP2B1, CYP2B2, CYP3A2 and CYP2C6 (figure 9). CYP1A1 was not detected (data not shown) and CYP1A2 was not changed by 3-$MeSO_2$-TetraBrB (figure 9).

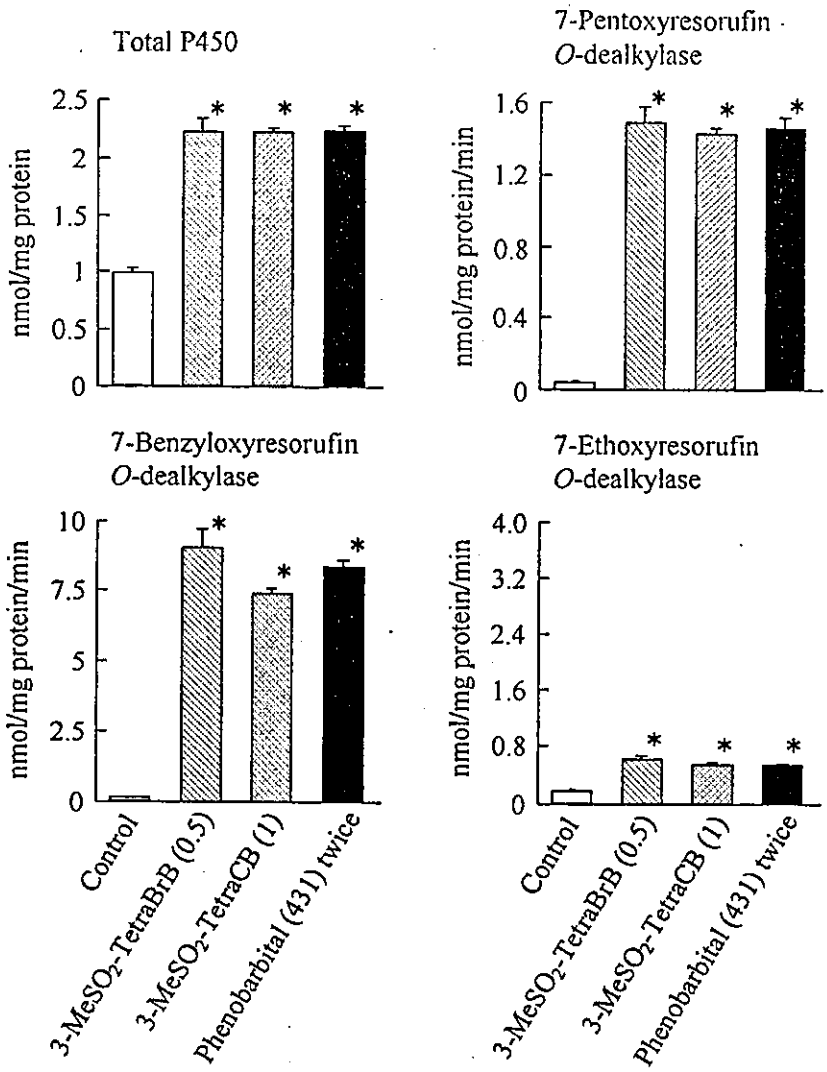

Figure 8. Effects of 3-MeSO$_2$-TetraBrB, 3-MeSO$_2$-TetraCB and phenobarbital on the content of total P450 and the activities of drug-metabolizing enzyme of rat liver microsomes. Rats were given 3-MeSO$_2$-TetraBrB or 3-MeSO$_2$-TetraCB (i.p.) and killed 96 h after administration. Phenobarbital was injected i.p. into rats twice with a 24-h interval and killed 24 h after the second injection. Results are the mean ± SEM for five to eight animals. The value in parentheses is a dose (μmol kg^{-1}) for each group. *$P < 0.001$, significantly different from the control.

Role of 3-MeSO$_2$-TetraBrB

Figures 10 and 11 show the relationship between the concentrations of 3- and 4-MeSO$_2$-TetraBrBs with the activities of drug-metabolizing enzymes, and with the contents of P450 forms in the liver after the administration of TetraBrB and its MeSO$_2$ derivatives to rat.

The extents of both the hepatic accumulation of the 3-MeSO$_2$ metabolite and the induction of P450, 7-pentoxy- and 7-benzyloxyresorufin *O*-dealkylases, and CYP2B1, CYP2B2, CYP3A2 and CYP2C6 after the administration of TetraBrB (342 μmol kg^{-1}) was almost the same as those after the administration of 3-MeSO$_2$-TetraBrB (0.1–0.2 μmol kg^{-1}). The 4-MeSO$_2$-TetraBrB metabolite (0.35 μmol kg^{-1}) had no effect on the content of P450, on the activities of the two alkoxyresorufin *O*-dealkylases and on the contents of PB-inducible forms of P450. Even 5 μmol kg^{-1} 4-MeSO$_2$-TetraBrB had no effect (data not shown).