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INHIBITION OF MONO-OXYGENASE ACTIVITIES BY 1,1,1-TRICHLOROPROPENE 2,3-OXIDE, AN INHIBITOR OF EPOXIDE HYDRASE, IN RAT LIVER MICROSOMES

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Abstract—Addition of 1,1,1-trichloropropene 2,3-oxide (TCPO), an inhibitor of microsomal epoxide hydrase, to rat liver microsomes caused a type I spectral change, and its magnitude was increased by pretreatment of animals with phenobarbital (PB) but not with 3-methylcholanthrene and polychlorinated biphenyls. TCPO inhibited aminopyrine *N*-demethylation competitively and prevented covalent binding of 2,4,2′,4′-tetrachlorobiphenyl to macromolecules catalyzed by liver microsomes, although it stimulated benzo[a]pyrene hydroxylation significantly. It is suggested that TCPO interacts with cytochrome P-450, especially a species of the cytochrome which is inducible by PB administration, and thus inhibits mono-oxygenase activities of liver microsomes.

Recent studies have shown that a variety of xenobiotics, including polychlorinated biphenyls (PCBs), are converted by the cytochrome P-450-linked mono-oxygenase system to active metabolites that are capable of covalently binding to intracellular macromolecules [1–4]. A number of reports have also indicated that arene oxides are the active metabolites formed from such chemicals as halogenated benzenes and polycyclic hydrocarbons [1,2,5–7]. The arene oxides thus produced can be readily hydrolyzed to inactive dihydrodiol derivatives by the action of a microsomal enzyme called epoxide hydrase (or hydratase) [7, 8]. Therefore, it is expected that inhibitors of epoxide hydrase will stimulate the covalent binding of xenobiotics which are convertible to arene oxides by the microsomal mono-oxygenase system. In fact, it has been shown that 1,1,1-trichloropropene 2,3-oxide (TCPO), a potent inhibitor of epoxide hydrase [8, 9], enhances covalent binding of benzo[a]pyrene and 3-methycholanthrene (3-MC) to DNA *in vitro* [10, 11] and potentiates 3-MC-induced carcinogenesis *in vivo* [12]. Relying upon these observations, TCPO has been used to study the involvement of arene oxide intermediates in cytotoxic effects induced by various drugs [13, 14]. However, the effect of TCPO on the microsomal mono-oxygenase system has not been fully explored [15, 16]. In this paper, we report evidence that TCPO interacts with cytochrome P-450 and thus inhibits drug oxidations catalyzed by the liver microsomal mono-oxygenase system. Among the multiple species of liver microsomal cytochrome P-450, the component inducible by phenobarbital (PB) pretreatment of animals seems to have the highest affinity for TCPO.

MATERIALS AND METHODS

$NADP^+$, glucose-6-phosphate, yeast glucose-6-phosphate dehydrogenase, and benzo[a]pyrene were purchased from the Sigma Chemical Co., St. Louis, MO, TCPO from the Aldrich Chemical Co., Milwaukee, WI, and two preparations of PCB mixtures, i.e. Kanechlor-300 * (KC-300; chlorine content, 42%) and Kanechlor-500 (KC-500; chlorine content, 55%), from the Kanegafuchi Kagaku Co., Tokyo. 2,4,2′,4′-Tetrachloro[^{14}C]biphenyl ([^{14}C]TCB; 3.8 mCi/mmole) was obtained from the Tokai Establishment of the Japan Atomic Energy Institute.

Male Sprague–Dawley rats, weighing 300–500 g, were allowed free access to a standard laboratory chow (Oriental Yeast Co., Tokyo) and water. They were killed under light ether anesthesia after starvation for 24 hr. The animals were also injected i.p. with PB (dissolved in saline; daily dose, 100 mg/kg body weight), 3-MC (dissolved in olive oil; 25 mg/kg), or KC-500 (dissolved in olive oil; 100 mg/kg) for 3 consecutive days, starved for 24 hr after the last injection, and killed as above. Liver microsomes were prepared as described by Omura and Sato [17].

Protein and cytochrome P-450 were determined by the methods of Lowry *et al.* [18] and Omura and Sato [17] respectively. Aminopyrine *N*-demethylation was measured in a reaction mixture of 1.0 ml, as described by Mazel [19]. Benzo[a]pyrene hydroxylation was assayed by a slight modification of the method of Nebert and Gelboin [20], as described previously [4]. Covalent binding of TCB metabolites to liver microsomal macromolecules was determined as follows. The reaction mixture (final volume, 1.0 ml) contained 0.1 M potassium phosphate buffer (pH 7.4), 0.87 μg [^{14}C]TCB (0.0114 μCi) dissolved in 10 μl acetone, microsomes (about 1 mg protein), and an NADPH-generating system consisting of 0.5 mM $NADP^+$, 10 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, and 3 mM $MgCl_2$. The reaction was run at 37° for 15 min and stopped by adding 3 ml of 10% trichloroacetic acid. The radioactivity in covalent linkages with microsomal macromolecules was determined as described previously [3, 4], and taken as a measure of covalent binding of TCB to microsomes.

* Kanechlor, KC (trade name of PCBs in Japan).

Spectrophotometric measurements were carried out at room temperature by using a Shimadzu MPS-50L multipurpose spectrophotometer.

RESULTS AND DISCUSSION

Figure 1 shows the spectral changes induced by the addition of hexobarbital, aniline, KC-300 and TCPO (each at 1 mM) to a suspension of liver microsomes from PB-pretreated rats. As reported previously [21–23], hexobarbital caused a typical type I difference spectrum having a peak at 385 nm and a trough at 422 nm, whereas aniline induced a type II spectrum with a peak at 427 nm and a trough at 392 nm. It is well established that these spectral changes are due to the binding of respective substrates to the oxidized form of cytochrome P-450. It will be seen that KC-300 and TCPO also caused type I spectral changes which were qualitatively almost identical with that induced by hexobarbital. The induction of a spectral change by KC-300 is not surprising, since we have shown previously that PCBs such as KC-300 can be metabolized by the cytochrome P-450-linked mono-oxygenase system and are thereby converted to active metabolites that are capable of covalently binding to microsomal macromolecules [3, 4]. Although TCPO has not yet been reported to be metabolizable by the mono-oxygenase system, the induction of a clear type I difference spectrum by this compound suggests that it can bind to oxidized cytochrome P-450.

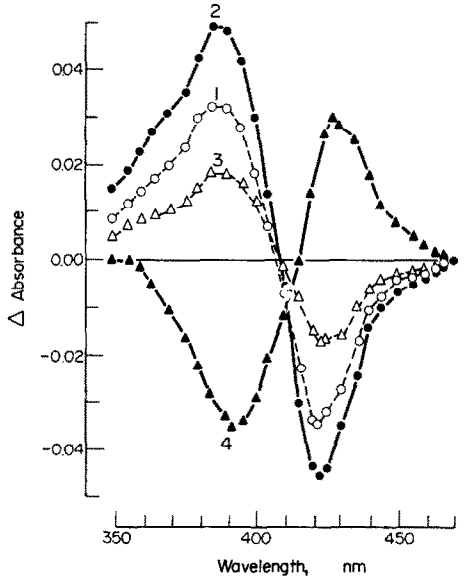

Fig. 1. Spectral changes caused by the addition of hexobarbital (curve 1), KC-300 (curve 2), TCPO (curve 3), and aniline (curve 4), each at a concentration of 1 mM, to a suspension of liver microsomes prepared from PB-pretreated rats. The microsomes were suspended in 0.1 M potassium phosphate buffer (pH 7.4) to a concentration of 1.0 mg protein/ml.

As shown in Table 1, liver microsomes prepared from control (drug-untreated) rats also exhibited a type I spectral change in response to TCPO addition, and its intensity was increased about 2-fold after pretreatment of rats with PB. Administration of 3-MC and KC-500, on the other hand, caused practically no increase in the intensity of TCPO-induced spectral change, in spite of the fact that all three pretreatments increased the cytochrome P-450 content in liver microsomes. The increases in cytochrome P-450 content caused by PB, 3-MC and KC-500 treatments were 2.6-, 1.7- and 2.1-fold respectively. Consequently, the magnitude of TCPO-induced spectral change per unit amount of cytochrome P-450 was considerably decreased after 3-MC and KC-500 treatments, whereas the decrease caused by PB treatment was not so significant. These results suggest that TCPO has a significantly higher affinity for cytochrome P-450 in PB-induced liver microsomes than for that in 3-MC- or KC-500-induced microsomes. The type I difference spectra caused by hexobarbital and KC-300 responded to the various pretreatments in a fashion similar to that induced by TCPO (Table 1), suggesting that those two compounds also interact preferentially with cytochrome P-450 in PB-induced microsomes. The type II spectrum caused by aniline, on the other hand, was intensified by pretreatments of rats with all three inducers, but the magnitude of aniline-induced spectral change per unit amount of cytochrome P-450 increased only after 3-MC administration (Table 1).

In agreement with previous findings [4, 24–26], the reduced CO difference spectra of liver microsomes from 3-MC- and KC-500-treated rats exhibited a Soret

Table 1. Effects of pretreatment of rats with PB, 3-MC or KC-500 on the spectral changes induced in liver microsomes by the addition of TCPO, hexobarbital, KC-300 or aniline*

Pretreatment	TCPO $\Delta A_{385-422\ nm}$	Hexobarbital $\Delta A_{385-422\ nm}$	KC-300 $\Delta A_{385-422\ nm}$	Aniline $\Delta A_{427-392\ nm}$
None (control)	0.016	0.035	0.033	0.024
	(0.019)	(0.042)	(0.039)	(0.029)
PB	0.034	0.066	0.092	0.062
	(0.015)	(0.030)	(0.041)	(0.028)
3-MC	0.017	0.026	0.025	0.053
	(0.012)	(0.018)	(0.017)	(0.036)
KC-500	0.018	0.019	0.040	0.051
	(0.010)	(0.011)	(0.023)	(0.029)

* Figures in parentheses indicate the intensity of spectral change per nmole of cytochrome P-450 (or P-448). Difference spectra were measure as described in Fig. 1 except that liver microsomes (1.0 mg protein/ml) prepared from the variously pretreated rats were used. TCPO, hexobarbital and KC-300 gave type I spectral changes, and aniline gave type II changes with all the microsomal preparations employed.

peak at 448 nm, whereas those of control and PB-induced microsomes exhibited a peak at 450 nm. Evidence is now available that the major cytochrome P-450 component in liver microsomes from 3-MC-treated animals, called cytochrome P-448 or P_1-450, is different from that of PB-induced liver microsomes [27, 28]. It has also been shown that pretreatment of rats with PCBs such as KC-500 induces the synthesis in liver microsomes of both cytochrome P-448 and the PB-inducible species of cytochrome P-450 [4, 24–26]. Although liver microsomes from control rats exhibit a Soret peak at 450 nm, it is not yet known if the major cytochrome P-450 in these microsomes is identical with the PB-inducible one.

Taking this multiplicity of liver microsomal cytochrome P-450 into consideration, it may be concluded that TCPO, hexobarbital and KC-300 all interact more efficiently with the PB-inducible species of cytochrome P-450 (and probably also with the major component in control microsomes) than with cytochrome P-448 which is present in 3-MC- and KC-500-induced microsomes. In fact, it has been reported that PB-induced liver microsomes metablolize hexobarbital much more actively than 3-MC-induced microsomes [29]. Our previous work [4] has also provided evidence that the PB-inducible cytochrome P-450 catalyzes the covalent binding of PCBs (KC-300 and KC-500) to microsomal macromolecules more efficiently than cytochrome P-448.

Since TCPO was found to interact with cytochrome P-450, especially with the species inducible by PB, it was of interest to examine the effects of this compound on the metabolism of various drugs by the microsomal mono-oxygenase system. For this purpose, aminopyrine *N*-demethylation, benzo[a]pyrene hydroxylation, and covalent binding of TCB metabolites to microsomes (T. Shimada and R. Sato, unpublished results) were chosen as representatives of drug-metabolizing activities. Table 2 summarizes the specific activities of these reactions in liver microsomes from control rats as well as in those from PB-, 3-MC- and KC-500-pretreated animals. As can be seen, PB treatment caused 2- and 10-fold increases in aminopyrine *N*-demethylase and TCB-binding activities, respectively, but had no effect on benzo[a]pyrene hydroxylase activity. 3-MC administration, on the other hand, resulted in a 3-fold increase in benzo[a]pyrene hydroxylase activity, but did not affect aminopyrine *N*-demethylase activity and caused only a 1.7-fold increase in TCB-binding activity. KC-500 treatment produced intermediate results, probably reflecting the aforementioned view that PCBs induce both PB-inducible cytochrome P-450 and 3-MC-inducible cytochrome P-448 [4, 24–26].

Figure 2 shows the effects of TCPO on the three drug-metabolizing activities of liver microsomes from the variously pretreated rats. It will be seen that the benzo[a]pyrene hydroxylase activities of both 3-MC- and KC-500-induced liver microsomes were activated about 1.5-fold by a low concentration of TCPO such as 0.01 mM, although 0.001 mM TCPO caused no activation (data not shown). Upon increasing the TCPO concentration, there was no further activation of the hydroxylase reaction, and at the concentration higher

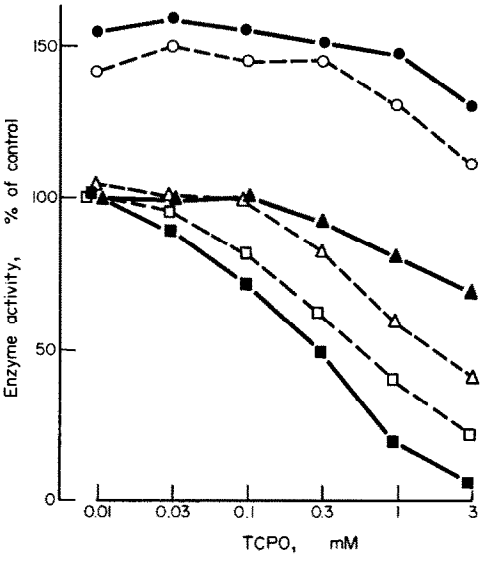

Fig. 2. Effects of TCPO on mono-oxygenase activities of liver microsomes from variously pretreated rats. The indicated final concentration of TCPO, dissolved in 20 μl dimethylsulfoxide, was added to the reaction mixture (1.0 ml); dimethylsulfoxide itself had little effect on the benzo[a]pyrene hydroxylase activity, but inhibited slightly aminopyrine *N*-demethylase and TCB binding activities by less that 15 per cent. The activities in the absence of TCPO were: 0.759 and 0.646 nmole/min/mg of protein for benzo[a]pyrene hydroxylation (3-hydroxybenzo[a]pyrene as reference standard) by 3-MC-treated (○) and KC-500-treated (●) liver microsomes, respectively; 6.03 and 5.29 nmoles/min/mg of protein for aminopyrine *N*-demethylation by PB-treated (△) and KC-500-treated (▲) microsomes, respectively; and 1596 and 915 dis./min/mg protein/15 min for TCB binding by PB-treated (■) and KC-500-treated (□) microsomes, respectively.

Table 2. Effects of pretreatment of rats with PB, 3-MC or KC-500 on liver microsomal mono-oxygenase activities*

Pretreatment	Aminopyrine *N*-demethylase activity (nmoles/mg protein/min)	Benzo[a]pyrene hydroxylase activity (nmoles/mg protein/min)	TCB binding activity (dis./min/mg protein/15 min)
None (control)	3.30	0.208	175
PB	6.98	0.225	1831
3-MC	3.12	0.648	297
KC-500	5.95	0.592	1040

* Pretreatment of rats with various inducers and determination of microsomal enzyme activities were performed as described in Materials and Methods. Values are the mean activities of liver microsomes from three rats.

than 1 mM a gradual decrease in the activity was observed. On the other hand, both the aminopyrine *N*-demethylase activity and the covalent binding of TCB metabolites to macromolecules catalyzed by liver microsomes from PB- and KC-500-treated rats were clearly inhibited by TCPO, and the inhibition was more pronounced with TCB-binding activity than with aminopyrine *N*-demethylase activity. Moreover, the two activities of PB-induced liver microsomes were more strongly inhibited by TCPO than those of KC-500-induced microsomes. This is in good agreement with the conclusion drawn above that TCPO has a higher affinity for the PB-inducible species of cytochrome P-450 than the other species of the hemoprotein. With PB-induced microsomes, 50 per cent inhibition of aminopyrine *N*-demethylase and TCB-binding activities was attained by 1.7 and 0.3 mM TCPO respectively.

Furthermore, as shown in Fig. 3, TCPO inhibited aminopyrine *N*-demethylation by PB-induced liver microsomes in a competitive manner.

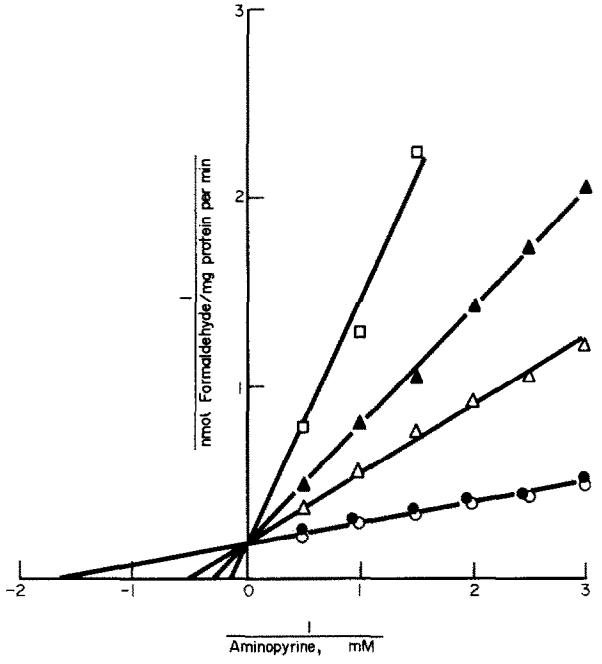

Fig. 3. Lineweaver–Burk plot of the effect of TCPO on the aminopyrine *N*-demethylation by liver microsomes from PB-pretreated rats. Key: (○) no addition; (●) dimethylsulfoxide (10 μl); (△) 1 mM TCPO; (▲) 3 mM TCPO; and (□) 9 mM TCPO.

The results described above indicate clearly that TCPO, in addition to its action as an inhibitor of epoxide hydrase, is capable of interacting with the oxidized form of cytochrome P-450 in liver microsomes, and thus causes a type I spectral change. It is also clear that this binding results in inhibition of microsomal mono-oxygenase activities, such as aminopyrine *N*-demethylation and formation of active TCB metabolites which can covalently bind to macromolecules. The effects of pretreatment of animals with various inducers on the TCPO-induced spectral change (Table 1) further suggest that TCPO has a much higher affinity for the PB-inducible species of cytochrome P-450 than the other species of the cytochrome. The cytochrome P-450 species which is the major component of liver microsomes from control (drug-untreated) rats also seems to interact with TCPO rather efficiently. As shown in Fig. 2, benzo[a]pyrene hydroxylase activities of 3-MC- and KC-500-induced liver microsomes are stimulated significantly by low concentrations (0.01 to 0.3 mM) of TCPO. Prough *et al.* [30] have also reported that the conversion of benzo[a]pyrene to phenolic metabolites by liver microsomes can be enhanced by 20–30 per cent in the presence of TCPO. The mechanism of this activation is not yet clear, but Fig. 2 shows that gradual inhibition of benzo[a]pyrene hydroxylation takes place when the TCPO concentration exceeds 0.1 mM, suggesting that the species of cytochrome P-450 (3-MC-inducible cytochrome P-448) responsible for this hydroxylation can also be inhibited by higher concentrations of TCPO.

We have reported previously that the cytochrome P-450-linked mono-oxygenase system of liver microsomes catalyzes the conversion of PCBs to active metabolites that are capable of covalently binding to intracelluar macromolecules and suggested the possibility that PCB epoxides are the active metabolites [4, 31]. If PCB epoxides are actually involved in the covalent binding, it is expected that TCPO activates, rather than inhibits, the binding of metabolites of PCBs, such as TCB. Figure 2 shows, however, that this is not the case; TCPO inhibits the TCB binding at all the concentrations tested. This indicates that the inhibitory effect of TCPO on the conversion of TCB to its epoxides is stronger than the protective action of the compound on TCB epoxides from degradation by epoxide hydrase.

The conclusion reached in the present study, that TCPO can inhibit mono-oxygenase activities catalyzed by the cytochrome P-450 system, indicates that care should be taken in using TCPO and other inhibitors of epoxide hydrase to study the involvement of arene oxides in cytotoxic effects of xenobiotics. This is especially so in view of the report by Mukhtar and Bresnick [32] that TCPO also inhibits glutathione transferase.

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