Biochemical Pharmacology, Vol. 41, No. 11, pp. 1691–1699, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

EVIDENCE FOR THE STABILITY AND CYTOCHROME P450 SPECIFICITY OF THE PHENOBARBITAL-INDUCED REDUCTIVE HALOTHANE–CYTOCHROME P450 COMPLEX FORMED IN RAT HEPATIC MICROSOMES

MAX T. BAKER,* MARIA T. VASQUEZ and CHAO-KUO CHIANG
Department of Anesthesia, University of Iowa, Iowa City, IA 52242, U.S.A.

(*Received* 27 *August*; *accepted* 14 *December* 1990)

Abstract—The hypothesis that the reduced spectral halothane–cytochrome P450 complex formed in rat hepatic microsomes is a stable cytochrome P450 specific species was examined. Comparisons of the cytochrome P450 inducers, phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN) and β-naphthoflavone (β-NF) showed that PB was the most effective inducer of the halothane–cytochrome P450 complex and the cytochrome P450 which liberates the halothane metabolites, 2-chloro-1,1-difluoroethene (CDE) and 2-chloro-1,1,1-trifluoroethane (CTE). However, the ratio of CDE produced to quantity of complex was found to be reduced 70–77% in these microsomes. A large portion of total microsomal cytochrome P450 was destroyed upon halothane reduction (up to 39%), yet the complexed cytochrome P450, particularly in microsomes from PB-treated animals, was resistant to the irreversible inactivation mechanisms of halothane reduction. The effects of reductive halothane metabolism on subsequent warfarin metabolism showed that 7-hydroxywarfarin formation from either (*R*)- or (*S*)-warfarin in microsomes from PCN-treated, PB-treated or untreated rats was highly susceptible to irreversible inhibition. In microsomes from PB-treated, but not PCN or untreated rats, the formation of one warfarin metabolite, 4′-hydroxywarfarin from (*R*)-warfarin, could be shown to be increased when complex was eliminated by photodissociation. These results suggest that PB-B is preferentially bound as complex and resistant to inactivation because of complex stability, and that halothane reduction readily destroys the cytochrome P450 form, PB-C.

In whole animals, hepatic microsomes and purified cytochrome P450 preparations, halothane ($CF_3CHClBr$) is reduced to 2-chloro-1,1,1-trifluoroethane (CTE) and 2-chloro-1,1-difluoroethene (CDE) [1–5]. These metabolites arise from the ability of halothane to accept either a single electron or two electrons sequentially from cytochrome P450 under low oxygen conditions [1, 6]. During the course of this metabolism, a spectral complex which absorbs light maximally at 470 nm, is formed between halothane and cytochrome P450 [1, 6, 7], and a portion of the cytochrome P450 is irreversibly inactivated [8, 9]. Cytochrome P450 inactivation is thought to be due to the release of a one electron reduced free radical, $CF_3CHCl\cdot$, which reacts with vital components of cytochrome P450, possibly the heme moiety [8–11]. This free radical is also responsible for the formation of CTE which occurs when it abstracts hydrogen from cellular constituents [6]. The complex, in contrast, has been identified as a two electron reduced halothane carbanion species bound to ferric heme iron ($CF_3CClH^-\cdots Fe^{3+}$) which spontaneously decays to release CDE by β-elimination of fluoride [1, 12, 13]. The latter finding has led to the hypothesis that the spectral complex is a steady-state precursor to CDE, and that its magnitude is indicative of the rate of CDE formation [1, 2].

The cytochrome P450 isozymes which carry out halothane reduction and, furthermore, whether each of these measures of halothane metabolism is preferentially performed by the same isozymes are unclear. Studies of halothane reduction in microsomes from animals treated with a variety of cytochrome P450 inducers, including phenobarbital, 3-methylcholanthrene and Arochlor [1], and in several purified cytochrome P450 forms [2, 4] showed that both CDE and CTE are produced in all cytochrome P450 preparations, and the metabolites are formed in similar ratios where CTE always predominates. Of the inducers examined, phenobarbital treatment resulted in the formation of the largest quantity of CDE and CTE, as well as complex [1]. It also resulted in the greatest degree of cytochrome P450 destruction [8]. Such evidence indicates that complex formation and cytochrome P450 inactivation are directly related to metabolite production, and all measures of halothane reduction are preferentially performed by one or more of the major forms of cytochrome P450 induced by phenobarbital.

While this appears to be the case, many metabolic cytochrome P450 complexes are stable entities which play either no role or a minor role in metabolite formation [14–16]. Even though studies have shown that in microsomes the halothane–cytochrome P450 complex decays to CDE, it has not been demonstrated that the rate of decay of the complex is sufficient to account for the quantity of CDE formed or that the complexed cytochrome P450 plays a major role in

* Correspondence: Max T. Baker, Ph. D., Department of Anesthesia, University of Iowa, Iowa City, IA 52242.

any aspect of halothane reduction other than complex formation. For those reasons, and the fact that phenobarbital induces several major forms of cytochrome P450 [17, 18], it is possible that the spectral halothane–cytochrome P450 complex induced by phenobarbital also represents a stable cytochrome P450 specific species which would exclude it as playing a major role in metabolite formation and cytochrome P450 inactivation.

The present study evaluated this hypothesis by investigating the relationships between complex formation and metabolite production, and cytochrome P450 inhibition in hepatic microsomes. This was done by (1) examining the involvement of induced cytochrome P450 forms in complex and metabolite formation, (2) determining the susceptibility of the complexed cytochrome P450 to inactivation, and (3) by assessing the effects of halothane reduction and complex on the metabolism of the cytochrome P450 specific probes, (*R*)- and (*S*)-warfarin [19–22]. The results support the hypothesis that the halothane complexed-cytochrome P450 is a low-turnover entity selective for cytochrome P450.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (180–210 g) were purchased from Amitech Inc. (Omaha, NE). The animals (four to five per group) were fed Teklad rat chow and were either untreated or treated with phenobarbital (0.2% sodium phenobarbital in the drinking water) for 4 days; pregnenolone-16α-carbonitrile (PCN) (75 mg/kg, i.p., in corn oil) for 3 days; or β-naphthoflavone (β-NF) (80 mg/kg, i.p., in corn oil) for 2 days. The animals were killed with CO_2, livers removed, and microsomes prepared by differential centrifugation of the liver homogenates in 1.15% KCl. Microsomes from the livers of rats in each group were pooled and frozen at −70° until used in a 0.1 M sodium phosphate buffer (pH 7.5) containing 1 mM EDTA.

Chemicals. Halothane and PCN were purchased from the Anaquest Corp. and the Upjohn Co., respectively. (*R*)- and (*S*)-Warfarin were gifts from Dr. Joel Whitney of the DuPont Co. The warfarin metabolites, 4′-hydroxy-, 6-hydroxy-, 7-hydroxy-, and 8-hydroxywarfarin were gifts from Dr. William Trager. Dehydrowarfarin was synthesized according to the method described by Fasco *et al.* [23], and its identity was verified by mass spectral analysis. CTE and CDE were purchased from PCR Chemicals.

Incubations. Reductive halothane incubations were performed in 9-mL glass hypovials sealed with Hycar Septa. They contained 3 or 4.5 mL of microsomal protein at 5 or 3 mg/mL in a 0.1 M sodium phosphate buffer, pH 7.4. Microsomal preparations were made hypoxic by flushing each vial containing the microsomes with prepurified nitrogen three times for 2 min each at a flow rate of approximately 2 L/min. Reducing equivalents for each incubation were supplied with an NADPH-generating system which was composed of 0.4 units glucose-6-phosphate dehydrogenase, 5 μmol NADP and 4 μmol glucose-6-phosphate. Halothane was added in liquid form, or as nitrogen gas saturated with halothane vapor at room temperature. Halothane vapor was added using a gas-tight syringe following withdrawal of a corresponding volume of headspace. Incubations were performed for the time periods indicated at 37° and stopped on ice.

Halothane complexed cytochrome P450 was assayed by measuring the absorbance difference in microsomes between 510 and 470 nm and using the extinction coefficient of 80 $cm^{-1}mM^{-1}$ determined by Ahr *et al.* [1]. Cytochrome P450 levels were determined by the method of Omura and Sato [24]. Cytochrome P450 not complexed to halothane (free cytochrome P450) was determined by placing the chilled halothane-incubated microsomes in both the reference and sample cuvette of the spectrophotometer and adding dithionite and carbon monoxide to those in the sample cuvette. These cytochrome P450 measurements were taken within 1 min after the addition of carbon monoxide. Total cytochrome P450 was measured following treatment of the microsomes with 1 mM $K_3Fe(CN)_6$ to eliminate the complex.

Measurement of reductive halothane metabolites. For the measurements of CTE and CDE formation, 1 mL of hexane was injected into the sealed vials after incubation. The vials were vortexed vigorously and centrifuged at approximately 500 *g* for 10 min to separate the phases. The vials were cooled in a dry-ice acetone bath (−78°), and the hexane phase was analyzed for CTE and CDE by gas chromatographic analysis. Gas chromatography was performed by injecting 10 μL of the hexane phase onto a 1/8 in. i.d. × 6 ft. column packed with Porapak Q. CTE and CDE were monitored with a flame ionization detector. These metabolites were identified and quantitated using authentic CTE and CDE.

Assay for warfarin metabolites following halothane reduction. To determine the effects of halothane reduction on warfarin metabolism, microsomes (3 mg/mL, 4.5 mL) were preincubated without and with halothane (1 μmol) under anaerobic conditions for 10 min as described above. After halothane preincubation, halothane and its volatile metabolites, CTE and CDE, were removed from the microsomes by placing the microsomes (4.5 mL) in 40-mL clear borosilicate glass hypovials which were sealed and covered with aluminium foil. These vials were placed in an ice bath and repeatedly evacuated and purged with nitrogen for a 30-min period (2-min cycles) using a Virtis lyophilizer as the vacuum source. To those microsomal preparations incubated without halothane, halothane was added after the incubation period but prior to evacuation as a control. For the microsomes in which the complex present was to be dissociated, microsomes were placed in uncovered vials and exposed to high-intensity white light during the evacuation and purging process. The vials were illuminated with a 300 W tungsten halogen lamp installed in a Kodak 800H Carousel Projector. This light source as used produced an irradiance of 30 W/m^2 at the surface of the vials as measured with a Yellow Springs Instrument model 65 Radiometer.

Following these treatments the microsomes (1 mL each) were placed in 25-mL Erlenmeyer flasks to which 0.5 mg of NADPH and (*R*)- or (*S*)-warfarin

sodium (1 mM) were added. Incubations were performed for 4 min at 37° under air and were stopped by the addition of 0.5 mL of acetone. Warfarin metabolites were extracted twice with 2 mL of ether:ethyl acetate (1:1) following the addition of 200 μL of 0.5 M NaH_2PO_4 [25]. Analysis of the warfarin metabolites was performed essentially by the HPLC method of Kaminsky *et al.* [20]. Metabolites were separated on an ISCO 5 μm C_{18} column (0.46 × 250 mm) using an isocratic solvent system of 25% acetonitrile in water containing 1.5% acetic acid at a pH of 4.95. The flow rate was 1.5 mL/min and metabolites were monitored at 313 nm. Metabolites were quantitated from standard curves constructed with authentic standards. The metabolite identified as 10-hydroxywarfarin by Kaminsky *et al.* [26] was not available; however, its identity was confirmed by its selective induction by PCN and by its relative retention time. The quantity of 10-hydroxywarfarin produced was estimated by the absorbance of warfarin at 313 nm.

Statistical analyses. Statistical analyses were performed by Student's *t*-test. P values of less than 0.05 were considered significant.

RESULTS

The effects of the cytochrome P450 inducers, β-NF, PCN, and phenobarbital, on the ability of microsomes to form complex, and to liberate CDE and CTE from halothane are shown in Table 1. β-Naphthoflavone was ineffective in inducing complex formation. However, PCN and phenobarbital did increase cytochrome P450 capable of complexing with halothane. Phenobarbital was a much more effective inducer of this cytochrome P450 where the quantity of complex was nearly three times greater than that induced by PCN and accounted for 36% of the total cytochrome P450.

β-Naphthoflavone also did not induce metabolite formation, but phenobarbital and PCN were potent inducers of CDE and CTE formation and were nearly equally as effective in doing so (Table 1). There were some differences in the metabolite formation, however. Microsomes from the PCN-treated rats produced slightly greater levels of CDE than those from phenobarbital-treated rats, but less CTE. This is reflected in a 1.6-fold rise in the CDE to CTE ratio for the PCN microsomes. The ratio of CDE produced to amount of complex in microsomes from PB-treated rats was only 23–30% of that in microsomes from untreated, or PCN-, or β-NF-treated rats.

The loss of total cytochrome P450 and halothane-complexed cytochrome P450 during the course of halothane reduction in microsomes from phenobarbital-treated rats is shown in Fig. 1. Total cytochrome P450 levels decreased to the extent of 39% during a 30-min incubation period. In contrast, the quantity of complex remained relatively constant decreasing only 9% from its highest level. At each time point the amount of complexed cytochrome P450 was approximately equal to the difference between the total and free cytochrome P450, confirming that the complexed cytochrome P450 remained intact during the 30-min incubation period.

Table 1. Effects of cytochrome P450 inducers on halothane complex and halothane reduction in rat hepatic microsomes*

Inducer	P450 (nmol/mg)	Complex (nmol/mg)	CDE (nmol/mg/15 min)	CTE	CDE/CTE	CDE/Complex
None	1.03	0.08	1.34 ± 0.05	3.47 ± 0.27	0.39	16.75
Phenobarbital	2.24	0.81	3.11 ± 0.18†	11.50 ± 0.55†	0.27	3.84
PCN	1.59	0.28	3.63 ± 0.11†	8.11 ± 0.52†	0.45	12.96
β-NF	1.75	0.08	0.98 ± 0.05†	3.17 ± 0.14	0.31	12.15

* Incubations contained 3 mL of microsomal protein (5 mg/mL) and 16.2 μmol halothane in 9-mL hypovials, and were incubated for 15 min. Values are means (or means ± SEM) of triplicate determinations.
† Significantly different ($P < 0.05$) from untreated animals.

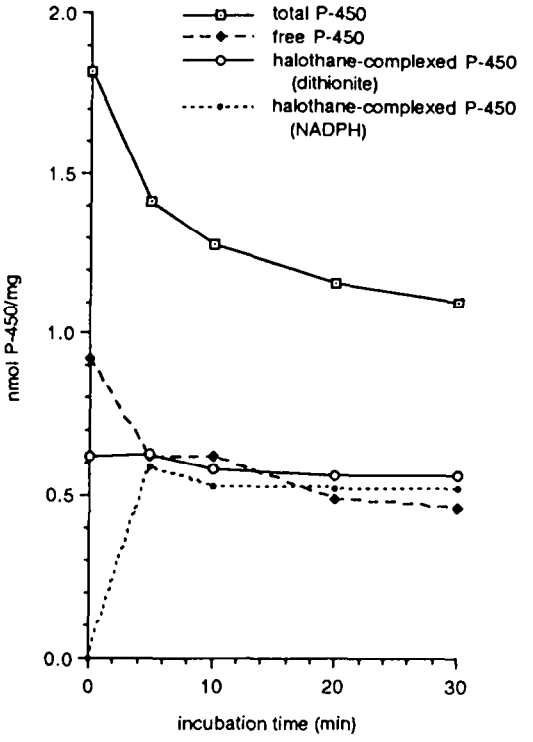

Fig. 1. Effects of halothane reduction on cytochrome P450 and halothane–cytochrome P450 complex levels in microsomes from phenobarbital-treated rats. Incubations contained 5 mg/mL microsomal protein (4.5 mL) and 16.2 μmol halothane. Incubations and analyses were performed as described in Materials and Methods.

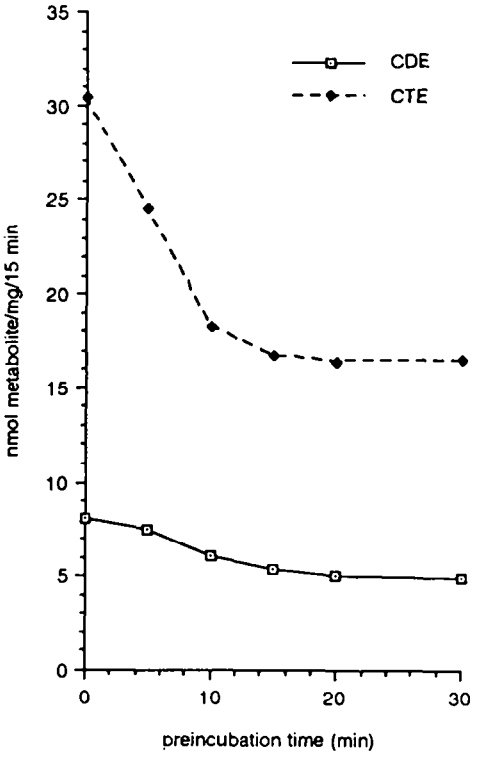

Fig. 2. Time course for the effects of halothane reduction on the capacity of microsomes from phenobarbital-treated rats to produce CDE and CTE. Incubations contained 5 mg/mL microsomal protein (3 mL) and 1 μmol halothane. Incubations and analyses were carried out as described in Materials and Methods.

The addition of dithionite to the microsomes following incubation had little effect on the level of complex, indicating that the complex was formed maximally in all incubations.

In microsomes from untreated rats, in similar 30-min halothane incubations, complex decreased from 0.05 ± 0.01 to 0.03 ± 0.01 nmol/mg protein (mean ± SEM of triplicate determinations), while total cytochrome P450 fell from 0.81 ± 0.05 to 0.70 ± 0.02 nmol/mg. In microsomes from PCN-treated rats, complex decreased from 0.22 ± 0.01 to 0.19 ± 0.02 (14% loss) and total cytochrome P450 decreased from 1.16 ± 0.02 to 0.62 ± 0.10 (46% loss).

The ability of halothane reduction to inhibit subsequent halothane metabolite formation in microsomes from the phenobarbital-treated animals is shown in Fig. 2. While there was little effect on complex levels during the 30-min incubation period, the capability of these microsomes to form CDE and CTE was impaired by 40 and 45%, respectively. Sixty-two percent of the inhibition of CDE production, and 87% of the inhibition of CTE formation occurred within the first 10 min of the preincubation period with halothane after which there was a substantially lower rate of loss of CTE and CDE production.

Effects of halothane reduction on warfarin metabolism. In an effort to distinguish the cytochrome P450 forms which complex with halothane from those which are inactivated, and in an effort to evaluate the possible inhibitory properties of the complex, the effects of halothane reduction and the presence of complex on warfarin metabolism were determined. To accomplished this, it was necessary to retain the complex during halothane removal, as well as to dissociate it without impairing cytochrome P450. This was done by evacuating and purging the microsomes with nitrogen while on ice for 30 min, and by photodissociating the complex with high-intensity white light. The stability of the complex during the evacuation procedure and the effectiveness of the light in photodissociating the complex are shown in Fig. 3. In microsomes from phenobarbital-treated rats, where the complex levels were the highest, complex decayed to the extent of only 5% during the 30-min evacuation and purging process alone. Exposure of these microsomes to high-intensity light for 30 min, on the other hand, resulted in a consistent loss of about 95% of the complex. In microsomes from untreated and PCN-treated animals, the high-intensity light likewise decreased the lesser amounts of complex. The evacuating and purging procedure was determined to remove halothane and its metabolites to nondetectable levels as assayed by gas chromatography described in Materials and Methods.

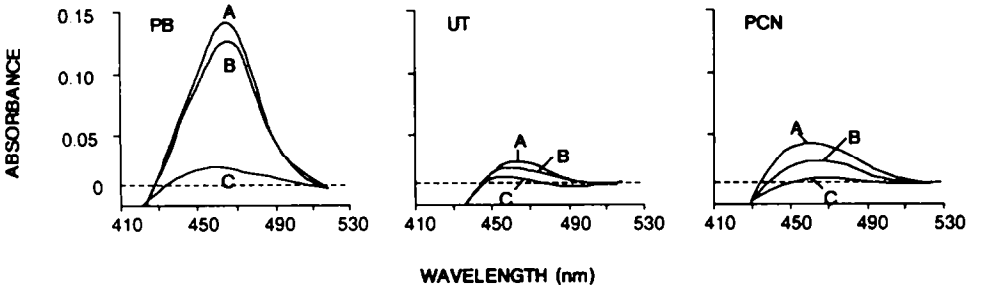

Fig. 3. Effects of evacuation and high-intensity light exposure on the halothane–cytochrome P450 complex in microsomes from untreated (UT), and phenobarbital (PB)- and pregnenolone-16α-carbonitrile (PCN)-treated rats. Key: (A) initial total complex; (B) complex following evacuation; and (C) complex following evacuation and light exposure.

Table 2. Effects of halothane reduction on the metabolism of (*R*)- and (*S*)-warfarin in hepatic microsomes from phenobarbital-treated rats*

Preincubation	No halothane (nmol/mg/min)	Halothane (nmol/mg/min)	(% Change)	Halothane, photodissociation (nmol/mg/min)	(% Change from halothane group)
(*R*)-Warfarin					
Dehydro-	0.59 ± 0.01	0.45 ± 0.01	(−24)	0.41 ± 0.01	(−9)
4′-Hydroxy-	0.36 ± 0.01	0.15 ± 0.01	(−58)	0.21 ± 0.01	(+40)
6-Hydroxy-	0.51 ± 0.01	0.19 ± 0.01	(−63)	0.19 ± 0.01	NS†
8-Hydroxy-	0.25 ± 0.01	0.10 ± 0.01	(−60)	0.11 ± 0.01	NS
10-Hydroxy-	4.40 ± 0.10	2.76 ± 0.01	(−37)	2.46 ± 0.10	(−11)
7-Hydroxy-	3.24 ± 0.04	0.39 ± 0.03	(−88)	0.33 ± 0.04	NS
(*S*)-Warfarin					
Dehydro-	1.66 ± 0.03	1.40 ± 0.02	(−16)	1.22 ± 0.01	(−13)
4′-Hydroxy-	0.32 ± 0.01	0.14 ± 0.01	(−56)	0.16 ± 0.01	NS
6-Hydroxy-	0.42 ± 0.01	0.19 ± 0.01	(−55)	0.19 ± 0.01	NS
8-Hydroxy-	0.16 ± 0.01	0.09 ± 0.01	(−44)	0.09 ± 0.01	NS
10-Hydroxy-	1.81 ± 0.02	0.88 ± 0.01	(−51)	0.82 ± 0.01	(−7)
7-Hydroxy-	0.69 ± 0.03	0.07 ± 0.01	(−90)	0.08 ± 0.01	NS

* Incubations were performed as described in Materials and Methods. Values are means ± SEM of triplicate determinations.

† NS, not significantly different from corresponding control group ($P > 0.05$).

Because microsome exposure to this light source itself could affect cytochrome P450 activity toward warfarin, this possibility was examined by exposing non-preincubated microsomes from phenobarbital-treated animals to the light for 30 min and examining their ability to metabolize (*R*)-warfarin. No statistically significant increases or decreases in formation of any of the metabolites examined in this study were observed due to light exposure alone (data not shown). Ambient room light was found to have no effect on the stability of the complex.

The effects of halothane reduction and the initial presence and absence of complexed cytochrome P450 on warfarin metabolism in microsomes from phenobarbital-treated animals are shown in Table 2. Preincubation of these microsomes with halothane for 10 min resulted in a loss of the ability of these microsomes to metabolize (*R*)- and (*S*)-warfarin to all monitored metabolites. However metabolite production was influenced selectively. The least affected was dehydrowarfarin whose formation was decreased 24 and 16% from (*R*)- and (*S*)-warfarin, respectively. 10-Hydroxywarfarin from (*R*)-warfarin was also minimally affected (37% loss). The metabolites, 4′-hydroxy- (58%), 6-hydroxy- (63%) and 8-hydroxy- (60%), from *R*-warfarin were intermediately susceptible to inhibition. These metabolites from (*S*)-warfarin were also affected to about the same degree: 4′-hydroxy- (56%), 6-hydroxy- (55%), and 8-hydroxy- (44%). 10-Hydroxywarfarin from (*S*)-warfarin was inhibited to a slightly greater degree than from (*R*)-warfarin, 51%. The metabolite most inhibited was 7-hydroxywarfarin whose production was impaired 88 and 90% from (*R*)- and (*S*)-warfarin, respectively.

When complex was photodissociated prior to warfarin incubation, the microsomes from the phenobarbital-treated rats had an increased capacity to form only one metabolite, 4′-hydroxywarfarin (+40%) from (*R*)-warfarin. Even though the

Table 3. Effects of halothane reduction on the metabolism of (*R*)- and (*S*)-warfarin in hepatic microsomes from untreated rats*

Preincubation	No halothane (nmol/mg/min)	Halothane (nmol/mg/min)	(% Change)	Halothane, photodissociation (nmol/mg/min)	(% Change from halothane group)
(*R*)-Warfarin					
Dehydro-	0.16 ± 0.01	0.13 ± 0.01	NS†	0.13 ± 0.01	NS
4′-Hydroxy-	0.33 ± 0.01	0.33 ± 0.01	NS	0.29 ± 0.01	(−12)
6-Hydroxy-	0.15 ± 0.02	0.14 ± 0.01	NS	0.12 ± 0.01	(−14)
8-Hydroxy-	0.08 ± 0.01	0.05 ± 0.01	(−38)	0.05 ± 0.01	NS
10-Hydroxy-	0.78 ± 0.01	0.72 ± 0.02	(−8)	0.55 ± 0.02	(−24)
7-Hydroxy-	0.47 ± 0.03	0.27 ± 0.01	(−43)	0.10 ± 0.02	(−63)
(*S*)-warfarin					
Dehydro-	0.40 ± 0.01	0.36 ± 0.01	(−10)	0.33 ± 0.01	NS
4′-Hydroxy-	0.35 ± 0.01	0.31 ± 0.02	NS	0.27 ± 0.01	NS
6-Hydroxy-	0.14 ± 0.01	0.13 ± 0.01	NS	0.12 ± 0.01	NS
8-Hydroxy-	0.07 ± 0.01	0.07 ± 0.01	NS	0.07 ± 0.01	NS
10-Hydroxy-	0.28 ± 0.01	0.23 ± 0.01	(−18)	0.19 ± 0.01	(−17)
7-Hydroxy-	0.09 ± 0.01	0.06 ± 0.01	(−33)	0.07 ± 0.01	NS

* Incubations were performed as described in Materials and Methods. Values are means ± SEM of triplicate determinations.

† NS, not significantly different from corresponding control group ($P > 0.05$).

formation of some metabolites was decreased slightly by light exposure of these microsomes preincubated with halothane, the production of no other metabolites from either (*R*)- or (*S*)-warfarin was increased by complex photolysis.

The effects of halothane preincubation on the metabolism of warfarin in microsomes from untreated rats are shown in Table 3. The total capacity of these microsomes to metabolize warfarin was not impaired to as great a degree by halothane as the phenobarbital-induced microsomes, and in fact not all metabolites were decreased significantly by halothane reduction. Halothane reduction, however, consistently decreased 7-hydroxywarfarin formation to the greatest degree, 43 and 33%, from (*R*)- and (*S*)-warfarin, respectively. There were no increases in 4′-hydroxywarfarin formation or other metabolites from either (*R*)- or (*S*)-warfarin following light exposure of the microsomes.

In microsomes from PCN-treated rats, 7-hydroxywarfarin production from (*R*)-warfarin was still impaired to the greatest degree (58%) (Table 4). From (*R*)-warfarin, the warfarin isomer that is more efficiently metabolized to 10-hydroxywarfarin [17], 10-hydroxywarfarin formation was impaired by only 25%. From (*S*)-warfarin, 10-hydroxywarfarin and 7-hydroxywarfarin production were inhibited to nearly the same extent, 49 and 47%, respectively. Like that in the microsomes from untreated rats, no increases in metabolite formation were observed due to photolysis of the complex in these microsomes.

DISCUSSION

Even though studies have shown that the microsomal halothane-cytochrome P450 complex which absorbs light at 470 nm decays to CDE, its participation in halothane reduction has not been clarified. This study presents several lines of evidence that at least a large portion of this complex is a cytochrome P450 specific entity, which rather than playing a major role, is involved to lesser degrees than other cytochrome P450 forms in the processes of halothane reduction. This is demonstrated by the finding that the quantity of CDE liberated based on the amount of complex was substantially lower in microsomes from phenobarbital-treated animals where the complex is highly induced. The complexed cytochrome P450, particularly in microsomes from phenobarbital-treated rats, was not irreversibly inactivated during the course of halothane reduction as was a large portion of the total cytochrome P450. Lastly, the production of a specific metabolite of warfarin, 4′-hydroxywarfarin from (*R*)-warfarin, could be shown to be reversibly inhibited by complex in the phenobarbital microsomes. Each of these findings can be explained by the fact that the halothane–cytochrome P450 complex, while not being a highly stable species [1, 13], is one of a sufficiently low turnover that it renders the bound cytochrome P450 resistant to inactivation, blocks the activity of the complexed cytochrome P450 even under oxidative metabolizing conditions, and does not allow as rapid metabolite liberation as other cytochrome P450 forms. Previous spectral measurements of complex decay in PB microsomes substantiate this conclusion. Decay of the complex to the extent of 80% from its maximum level required approximately 20–30 min at 37° [13] which indicates a turnover rate several times lower than that which can account for the rate of CDE formed in this and other microsomal studies [1, 3].

Evidence as to the cytochrome P450 forms preferentially involved in the different aspects of halothane reduction can be estimated from the effects of halothane reduction and complex on warfarin metabolism. That 4′-hydroxywarfarin formation from (*R*)-warfarin is the only metabolite

Table 4. Effects of halothane reduction on the metabolism of (*R*)- and (*S*)-warfarin in hepatic microsomes from PCN-treated rats*

Preincubation	No halothane (nmol/mg/min)	Halothane (nmol/mg/min)	(% Change)	Halothane, photodissociation (nmol/mg/min)	(% Change from halothane group)
(*R*)-Warfarin					
Dehydro-	0.54 ± 0.03	0.44 ± 0.04	NS†	0.48 ± 0.01	NS
4′-Hydroxy-	0.33 ± 0.03	0.29 ± 0.01	NS	0.31 ± 0.01	NS
6-Hydroxy-	0.21 ± 0.01	0.15 ± 0.01	(−29)	0.17 ± 0.01	NS
8-Hydroxy-	0.06 ± 0.01	0.06 ± 0.01	NS	0.05 ± 0.01	(−17)
10-Hydroxy-	3.51 ± 0.25	2.63 ± 0.12	(−25)	2.57 ± 0.01	NS
7-Hydroxy-	0.24 ± 0.10	0.10 ± 0.02	(−58)	0.05 ± 0.02	(−50)
(*S*)-Warfarin					
Dehydro-	0.95 ± 0.03	0.82 ± 0.03	(−14)	0.75 ± 0.01	(−9)
4′-Hydroxy-	0.44 ± 0.02	0.32 ± 0.01	(−27)	0.33 ± 0.01	NS
6-Hydroxy-	0.34 ± 0.01	0.21 ± 0.01	(−38)	0.19 ± 0.01	NS
8-Hydroxy-	0.12 ± 0.01	0.09 ± 0.01	(−25)	0.09 ± 0.01	NS
10-Hydroxy-	2.20 ± 0.09	1.13 ± 0.03	(−49)	1.00 ± 0.04	(−13)
7-Hydroxy-	0.15 ± 0.02	0.08 ± 0.01	(−47)	0.06 ± 0.01	NS

* Incubations were performed as described in Materials and Methods. Values are means ± SEM of triplicate determinations.

† NS, not significantly different from corresponding control group ($P > 0.05$).

from either (*R*)- or (*S*)-warfarin inhibited by complex, and that this reversible inhibition could only be demonstrated in microsomes from PB-treated rats, suggests that PB-B (P450IIB1) [27–29] is the isozyme predominantly bound as complex. PB-B, the major phenobarbital inducible cytochrome P450 form, selectively catalyzes 4′-hydroxywarfarin formation from (*R*)-warfarin, but not (*S*)-warfarin [17]. Other support for a major role of PB-B in halothane complex formation is from studies of complex in the purified cytochrome P450 forms, PBRLM5 (PB-B), PBRLM4 (PB-C, P450IIC6) [18, 27] and RLM2 which showed that PBRLM5 formed the greatest amount of complex; RLM2, very low quantities; and PBRLM4, none [2]. The isozyme(s) induced by PCN is also excluded as the form which preferentially binds as complex from the finding that PCN, a potent selective inducer of PB-PCN/E [17, 29], was capable of inducing only about 27% of the complex induced by phenobarbital. The lesser complex induction by PCN may be accounted for by the slight, but significant degree of PB-B induction by PCN [17]. An inability of complex dissociation in the PCN microsomes to result in a recovery of 4′-hydroxywarfarin metabolism is likely due to the lower quantities of complex in these microsomes.

The consideration that the complexed cytochrome P450 is a stable cytochrome P450 specific metabolic intermediate means that other phenobarbital-inducible forms of cytochrome P450 must be more susceptible to the irreversible inactivation mechanisms and be responsible for a major portion of the CDE and CTE liberated. Evidence that this is true for cytochrome P450 inactivation is provided by the finding that in microsomes from untreated, PCN-treated, and PB-treated rats, the formation of 7-hydroxywarfarin from (*R*)- or (*S*)-warfarin is the activity most susceptible to irreversible inhibition. 7-Hydroxywarfarin production from both (*R*)- and (*S*)-warfarin is an activity attributed to the isozyme PB-C [17].

An indication that the cytochrome P450 which liberates a large fraction of the reductive metabolites is distinct from that bound as complex, and also distinct from PB-C, is shown by the ability of PCN to effectively induce the cytochrome P450 which liberates CDE and CTE while it weakly induces complex. Indeed, as shown here, PCN induces CDE production to slightly greater levels than phenobarbital. The significance of this is that immunological estimations of individual cytochrome P450 forms in rat hepatic microsomes have shown that PB is an effective inducer of the isozyme(s) induced by PCN, PB-PCN/E; however, PCN does not induce the phenobarbital-inducible isozymes, PB-C and PB-D (P450IIB2), and it induces PB-B to only very low levels [17].

The time course for the effects of halothane reduction on halothane metabolite formation in the PB-induced microsomes appears to correlate with the differing roles of PB-C and PB-PCN/E in halothane reduction. In particular, there was a diphasic inhibition with time, especially evident for CTE formation, where 87% of the inhibition of the CTE formed occurred in approximately 10 min followed by a resistant phase. These microsomes also retained their ability to produce a majority of the CDE (55%) and CTE (60%) initially liberated even after 30 min. The rapid loss of metabolism is consistent with the loss of PB-C, since 7-hydroxywarfarin formation is inhibited nearly completely (90%) in a 10-min incubation period. The metabolism more resistant to loss may be due to PB-PCN/E with some contribution by PB-B and possibly other forms. An involvement of PB-PCN/E in the activity resistant to inactivation is supported not only by a major role of PB-PCN/E in metabolite formation indicated by PCN induction, but by the

finding that 10-hydroxywarfarin formation, the warfarin activity preferentially carried out by PB-PCN/E [17], is among the warfarin activities least susceptible to inhibition by halothane reduction. Since the other major isozyme induced by phenobarbital, PB-D, is a low activity form of cytochrome P450 not known to be specifically involved in warfarin metabolism, its role in complex formation or its susceptibility to inactivation could not be evaluated. Studies are needed to further clarify the specificities of the cytochrome P450 isozymes in halothane reduction especially in the light of the fact that PCN is known to induce at least two cytochrome P450 forms (P450III1, P450III2) [18, 29].

In conclusion, this study demonstrated that the spectral halothane–cytochrome P450 complex exhibits the properties of a stable, cytochrome P450 specific entity which is formed primarily with PB-B and which plays a lesser role in halothane reduction compared to other cytochrome P450 isozymes. For this reason the complex is not an index of either total halothane reduction or CDE liberation. Furthermore, due to the inhibitory properties of the complex, the effects of halothane reduction on cytochrome P450 activities can be concluded to occur by both irreversible and reversible processes selective for cytochrome P450.

REFERENCES

1. Ahr HJ, King LJ, Nastainczyk W and Ullrich V, The mechanism of reductive dehalogenation of halothane by liver cytochrome P-450. *Biochem Pharmacol* **31**: 383–390, 1982.
2. Van Dyke RA, Baker MT, Jansson I and Schenkman J, Reductive metabolism of halothane by purified cytochrome P-450. *Biochem Pharmacol* **37**: 2357–2361, 1988.
3. Baker MT and Van Dyke RA, Reductive halothane metabolite formation and halothane binding in rat hepatic microsomes. *Chem Biol Interact* **49**: 121–132, 1984.
4. Tamura S, Kawata S, Sugiyama T and Tarui S, Modulation of the reductive metabolism of halothane by microsomal cytochrome b_5 in rat liver. *Biochim Biophys Acta* **926**: 231–238, 1987.
5. Cousins MJ, Sharp JH, Gourlay GK, Adams JF, Haynes WD and Whitehead R, Hepatotoxicity and halothane metabolism in an animal model with application for human toxicity. *Anaesth Intens Care* **7**: 9–24, 1979.
6. Baker MT, Nelson RM and Van Dyke RA, The release of inorganic fluoride from halothane and halothane metabolites by cytochrome P-450, hemin, and hemoglobin. *Drug Metab Dispos* **11**: 308–311, 1983.
7. Mansuy D, Nastainczyk W and Ullrich V, The mechanism of halothane binding to microsomal cytochrome P-450. *Naunyn Schmiedebergs Arch Pharmacol* **285**: 315–324, 1974.
8. Krieter PA and Van Dyke RA, Cytochrome P-450 and halothane metabolism. Decrease in rat liver microsomal P-450 *in vitro*. *Chem Biol Interact* **44**: 219–235, 1983.
9. De Groot H, Harnisch U and Noll T, Suicidal inactivation of microsomal cytochrome P-450 by halothane under hypoxic conditions. *Biochem Biophys Res Commun* **107**: 885–891, 1982.
10. Trudell JR, Bosterling B and Trevor AJ, Reductive metabolism of halothane by human and rabbit cytochrome P-450. Binding of 1-chloro-2,2,2-trifluorethyl radical to phospholipids. *Mol Pharmacol* **21**: 710–717, 1982.
11. De Groot H and Noll T, Halothane hepatotoxicity: Relation between metabolic activation, hypoxia, covalent binding, lipid peroxidation and liver cell damage. *Hepatology* **3**: 601–606, 1983.
12. Ruf HH, Ahr H, Nastainczyk W, Ullrich V, Mansuy D, Battioni JP, Montiel-Montoya R and Trautwein A, Formation of a ferric carbanion complex from halothane and cytochrome P-450: Electron spin resonance, electronic spectra, and model complexes. *Biochemistry* **23**: 5300–5306, 1984.
13. Baker MT, Bates JN and Van Dyke RA, Stabilization of the reduced halocarbon–cytochrome P-450 complex of halothane by *n*-alkanes. *Biochem Pharmacol* **36**: 1029–1034, 1987.
14. Wrighton SA, Maurel P, Schuetz EG, Watkins PB, Young B and Guzelian PS, Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome $P\text{-}450_p$. *Biochemistry* **24**: 2171–2178, 1985.
15. Laignelet L, Narbonne J-F, Lhuguenot J-C and Riviere J-L, Induction and inhibition of rat liver cytochrome(s) P-450 by an imidazole fungicide (prochloraz). *Toxicology* **59**: 271–284, 1989.
16. Reidy GF and Murray M, Evidence for complexation of P-450 IIC6 by an orphenadrine metabolite. *Biochem Biophys Res Commun* **166**: 772–779, 1990.
17. Guengerich FP, Dannan GA, Wright ST, Martin MV and Kaminsky LS, Purification and characterization of liver microsomal cytochromes P-450: Electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**: 6019–6030, 1982.
18. Nebert DW, Adesnik M, Coon M, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Phillips IR, Sato R and Waterman MR, The P450 gene superfamily: Recommended nomenclature. *DNA* **6**: 1–11, 1987.
19. Kaminsky LS, Warfarin as a probe of cytochromes P-450 function. *Drug Metab Rev* **20**: 479–487, 1989.
20. Kaminsky LS, Fasco MJ and Guengerich FP, Production and antibodies to rat liver cytochrome P-450. *Methods Enzymol* **74**: 262–272, 1981.
21. Halpert J, Balfour C, Miller NE, Morgan ET, Dunbar D and Kaminsky LS, Isozyme selectivity of the inhibition of rat liver cytochromes P-450 by chloramphenicol *in vivo*. *Mol Pharmacol* **28**: 290–296, 1985.
22. Kaminsky LS, Guengerich FP, Dannon GA and Aust SD, Comparisons of warfarin metabolism by liver microsomes of rats treated with a series of polybrominated biphenyl congeners and by the component-purified cytochrome P-450 isozymes. *Arch Biochem Biophys* **225**: 398–404, 1983.
23. Fasco MJ, Dymerski PP, Wos JD and Kaminsky LS, A new warfarin metabolite: Structure and function. *J Med Chem* **21**: 1054–1059, 1978.
24. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes: Evidence for its protein nature. *J Biol Chem* **239**: 2379–2385, 1964.
25. Bush ED, Low LK and Trager WF, A sensitive and specific stable isotope assay for warfarin and its metabolites. *Biomed Mass Spectrom* **10**: 395–398, 1983.
26. Kaminsky LS, Dunbar DA, Wang PP, Beaune P, Larrey D, Guengerich FP, Schnellmann RG and Sipes IG, Human hepatic cytochrome P-450 composition as probed by *in vitro* microsomal metabolism of warfarin. *Drug Metab Dispos* **12**: 470–477, 1984.

27. Schenkman JB, Favreau LV, Mole J, Kreutzer DL and Jansson I, Fingerprinting rat liver microsomal cytochromes P-450 as a means of delineating sexually distinctive forms. *Arch Toxicol* **60**: 43–51, 1987.
28. Waxman DJ, Interactions of hepatic cytochromes P-450 with steroid hormones. Regioselective and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem Pharmacol* **37**: 71–84, 1988.
29. Graves PE, Kaminsky LS and Halpert J, Evidence for functional and structural multiplicity of pregnenolone-16α-carbonitrile-inducible cytochrome P-450 isozymes in rat liver microsomes. *Biochemistry* **26**: 3887–3894, 1987.