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REDUCTIVE METABOLISM OF HALOTHANE BY PURIFIED CYTOCHROME P-450*

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Abstract—The reductive metabolism of halothane was determined using purified RLM2, PBRLM4 and PBRLM5 forms of rat liver microsomal cytochrome P-450. The metabolites, 2-chloro-1,1,1-trifluoroethane (CTE) and 2-chloro-1,1-difluoroethylene (CDE), were determined. All three forms of cytochrome P-450 produced CTE with relatively small differences in its production among the various forms. There were major differences, however, in the production of CDE, with PBRLM5 being the most active. PBRLM5 was also the only form to show the development of a complex between halothane and cytochrome P-450. This complex absorbed light maximally at 470 nm. The complex formation and the production of CDE by PBRLM5 were stimulated by the addition of cytochrome b_5. Cytochrome b_5 had no effect on CDE production by PBRLM4 and inhibited the production of both CTE and CDE by RLM2. These results show that the two-electron reduction of halothane by cytochrome P-450 was catalyzed by the PBRLM5 form and that cytochrome b_5 stimulated the transfer of the second electron to halothane through PBRLM5, but not RLM2 or PBRLM4.

It is well known that the hepatic microsomal mixed-function oxidase system carries out the oxidation of a large number of endogenous and exogenous substrates [1–3]. This lack of specificity is evident because (1) cytochrome P-450, the terminal electron acceptor and substrate binding site of this mixed-function oxidase system, exists in multiple forms, (2) these cytochrome P-450 isozymes are able to metabolize many types of compounds, and (3) isozymes can be regulated nonuniformly by treatment of animals with microsomal enzyme inducers [4]. As a consequence of metabolism of certain substrates, this enzyme system also serves as a route of metabolic activation resulting in reactive metabolites that are suspected to play roles in toxic and carcinogenic events [5]. In addition to their role in the oxidation of a variety of substrates, cytochromes P-450 in the absence of oxygen catalyze the reduction of certain substrates, with the best examples being halogenated hydrocarbons [6–8], the reduction of which results in the detoxification as well as the metabolic activation of these substrates, e.g. halothane [9–11].

Halothane undergoes either oxidation or reduction by cytochrome P-450 depending on the concentration of oxygen present [12]. Both the oxidative and reductive metabolism have been associated with the hepatotoxicity of halothane [13, 14]. Since the reductive metabolism is more complex than the oxidative metabolism, considerable attention has been focused on the products of this metabolism. The products of the reductive reaction have been identified and quantitated, with two metabolites, 2-chloro-1,1,1-trifluorethane (CTE) and 2-chloro-1,1-difluoroethylene (CDE), being identified as the major reductive metabolites [15, 16]. It has been reported that a metabolite of halothane also binds to lipids, proteins and cytochrome P-450 [9]. The lipid binding has been described as a free radical reaction involving 2-chloro-1,1,1-trifluoroethyl free radical, which results from a one-electron reduction of halothane [17]. Binding of halothane to protein is suggested to be the reaction of a trifluoroacyl chloride intermediate with protein. In contrast, the halothane metabolite bound to cytochrome P-450 has been described recently as a two-electron reduced intermediate of halothane complexed with cytochrome P-450 which, upon decay of the complex, releases CDE accompanied by the beta-elimination of fluoride [18]. Since microsomes from phenobarbital-treated rats have been shown to be most active in formation of this complex [18], we used two forms of cytochrome P-450 from phenobarbital-treated rats, PBRLM4 and PBRLM5. For comparison, RLM2 from untreated rats was also included. The activity of the latter form was unaffected by cytochrome b_5, whereas the other two forms showed increased activity. This halothane metabolite–cytochrome P-450 complex can be detected spectrally by a strong absorption at 470 nm [19, 20] and has been stabilized for study [18]. The present study suggests that a specific form of cytochrome P-450 is responsible for the two-electron reduction of halothane and that the complex formation and subsequent CDE release are associated with the transfer of the second electron in which cytochrome b_5 plays a facilitative role.

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METHODS

Purification of cytochrome P-450 enzymes. Cytochrome P-450 enzymes were purified from hepatic microsomes of 200 g male CD rats (Charles River Breeding Laboratories, Wilmington, MA). The animals were either untreated (for RLM2 purification) or treated with phenobarbital by intraperitoneal injection (80 mg/kg) for 4 days (for the purification of PBRLM4 and PBRLM5). Previously described purification of cytochrome P-450 forms PBRLM4 and PBRLM5 was carried out by chromatographic procedures following solubilization of the microsomal preparation [21]. Cytochrome P-450 form RLM2 was purified as described by Jansson *et al.* [22]. NADPH–cytochrome P-450 reductase was isolated from microsomes from phenobarbital-pretreated rats according to Yasukochi and Masters [23], and cytochrome b_5 was purified from untreated rats as described [24]. Protein was determined by the method of Lowry *et al.* [25] and cytochrome P-450 by the method of Omura and Sato [26].

Cytochrome P-450 reconstitution. Reconstitution of mixed-function oxidase activity was performed as follows: Dilauroylphosphatidylcholine (DLPC, 8 μmol) was suspended in 1 ml of 50 mM sodium phosphate buffer, pH 7.25, containing 25% glycerol, 0.1 M NaCl and 5 mM EDTA. The suspension was clarified by sonication in a bath sonicator. This procedure yields vesicles of 20–25 nm diameter, as measured by electron microscopy [27]. Cytochrome P-450 (1.5 nmol) and NADPH–cytochrome P-450 reductase (1.5 nmol) were added to 250 nmol of DLPC at room temperature. When cytochrome b_5 was incorporated, 1.5 nmol was added. After 30 min at room temperature, this mixture was diluted with 50 mM sodium phosphate, pH 7.25, containing 25% glycerol to the appropriate volume for a final concentration of 1.5 μM cytochrome P-450 during the assay.

Each incubation consisted of 1 ml of the incubation mixture in a 6-ml hypovial. For metabolism, an NADPH-generating system, consisting of 1.8 nmol glucose-6-phosphate, 0.8 units glucose-6-phosphate dehydrogenase, and 1.8 nmol NADP or approximately 2 mg of crystalline sodium dithionite, was added to each vial. Blank incubations contained no reducing system. Prior to the addition of [^{14}C]halothane, each vial was flushed with pre-purified nitrogen at a flow rate of approximately 1 liter/min four times for 30 sec each. The vials were then sealed, and 10 μl of [^{14}C]halothane (New England Nuclear Corp.) in ethanol (approximately 0.5 μCi/10 μl) was injected. The mixtures were incubated for 30 min at 37° in a shaking water bath, and the reaction was stopped by adding 1 ml hexane to each incubation and placing on ice.

Spectrophotometric determinations. Spectra were determined with a Shimadzu UV 3000 spectrophotometer. Cytochrome P-450 preparations were identical to those used for metabolism except that non-radiolabeled halothane (0.22 μmol/ml) was added to the sample cuvette. Reductive halothane–cytochrome P-450 complex formation was determined by scanning between 400 and 525 nm in preparations containing sodium dithionite as reductant or following incubation for 10 min at 37° in those containing the NADPH-generating system.

Reductive halothane metabolite assays. Analysis of the formation of the reductive halothane metabolites, CTE and CDE, was carried out with reverse phase HPLC as previously described [16]. Following incubation, 1 ml of hexane was injected into each vial which was then vortexed and centrifuged (500 *g*). Prior to analysis, each vial was cooled to −78° with a dry ice–acetone bath. Twenty-five microliters of the hexane phase was withdrawn for immediate HPLC analysis. Metabolite separation was carried out on a reverse phase HPLC column (mobile phase, 50% methanol) utilizing a flow rate of 1 ml/min. Eluate fractions were collected at −78° and analyzed for radioactivity.

RESULTS

The metabolism of halothane to CTE and CDE was determined using the purified constitutive cytochrome P-450 enzyme RLM2 and two forms from phenobarbital-treated rats, PBRLM4 and PBRLM5. The recovery of halothane and its metabolites by the HPLC method was determined to be approximately 95%, using the cold trapping procedure. Table 1 shows the production of CTE and CDE in anaerobic incubations reduced either by the NADPH–cytochrome P-450 reductase system or by sodium dithionite using PBRLM5, the major phenobarbital-

Table 1. Reductive metabolism of halothane by cytochrome P-450 from PBRLM5

Addition	CTE	CDE	Halothane recovered
	(nmol/incubation)		
Sodium dithionite	2.12 ± 0.10	1.64 ± 0.10	122 ± 8
NADPH-generating system	2.38 ± 0.96	0.96 ± 0.07	110 ± 5
NADPH-generating system + cytochrome b_5	2.30 ± 0.18	2.10 ± 0.13	126 ± 11

Each incubation consisted of 1 ml of the incubation mixture in a 6-ml hypovial. For metabolism, an NADPH-generating system or crystalline sodium dithionite was added to each vial. Blank incubations contained no reducing system. See Methods for details of the formation of the reductive halothane metabolites, CTE and CDE.

Values are means ± SE, N = 4.

Table 2. Reductive metabolism of halothane by purified forms of cytochrome P-450

Cytochrome P-450 form	Cytochrome b_5	CTE (nmol/incubation)	CDE (nmol/incubation)	Halothane (nmol/incubation)	CTE/CDE
PBRLM5	−	1.94 ± 0.07	0.75 ± 0.06	404 ± 9	2.59
PBRLM5	+	1.46 ± 0.03	1.78 ± 0.13	236 ± 7	0.82
PBRLM4	−	2.23 ± 0.38	0.19 ± 0.16	323 ± 6	11.74
PBRLM4	+	2.89 ± 0.36	0.29 ± 0.05	354 ± 15	9.97
RLM2	−	1.23 ± 0.27	0.83 ± 0.12	301 ± 6	1.48
RLM2	+	0.71 ± 0.04	0.004 ± 0.04	254 ± 20	177.50

See Methods for details of incubation. All experiments were performed in the presence of an NADPH-generating system. Values are means ± SE, N = 4.

induced cytochrome P-450 [21]. Previous studies using hepatic microsomes have revealed a greater production of CDE by dithionite-reduced microsomes than that produced when the microsomes are reduced by NADPH [16]. Table 1 shows this to be the case with PBRLM5. However, when cytochrome b_5 was included with the PBRLM5, and the system reduced by NADPH, the CDE production was greater than that found when dithionite was the reductant but, more importantly, the activity was doubled when compared with the absence of cytochrome b_5 but reduced with NADPH. On the other hand, it should be noted that, while there was a change in CDE production, cytochrome b_5 inclusion had very little effect on CTE production. Cytochrome b_5 itself, reduced with dithionite or NADPH, did not produce CDE (data not shown).

Table 2 contains data of the CTE and CDE production with all three forms of cytochrome P-450. Production of the two metabolites and their ratios varied with the P-450 enzyme used. There were some differences in the substrate contents between the assays in Table 1 and those in Table 2. However, the relative differences within each assay remained the same. PBRLM5 showed the greatest stimulation in CDE production by the addition of cytochrome b_5. In contrast, RLM2 showed a production of CDE similar to that of PBRLM5 but with almost total inhibition of CDE production in the presence of cytochrome b_5. Further, the production of CTE by RLM2 was lower than that produced by the other forms.

Figure 1A is a halothane difference spectrum of reduced PBRLM5 showing a strong absorption maximum at 470 nm. This spectrum has been the subject of a previous publication and reflects the two-electron reduced halothane fragment associated (complexed) with cytochrome P-450 [18]. As shown in Fig. 1B, the magnitude of this complex was enhanced by the addition of cytochrome b_5. RLM2 and PBRLM4 did not produce significant amounts of this complex.

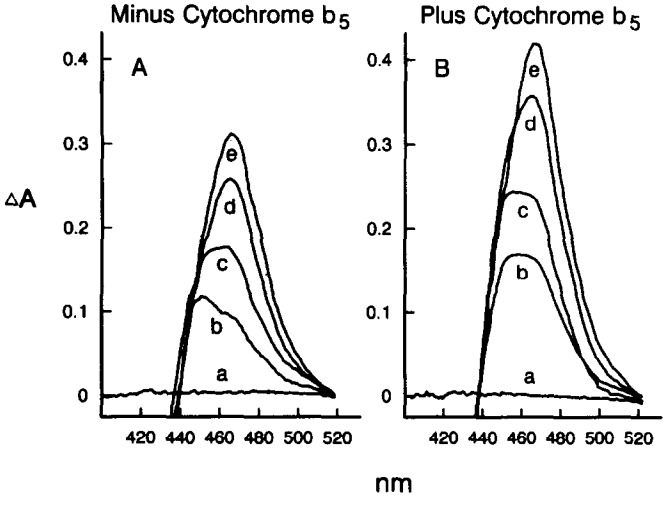

Fig. 1. Halothane–cytochrome P-450 complex formation in the presence and absence of cytochrome b_5. Complex formation was determined by scanning between 400 and 525 nm. (A) Sample and reference cuvettes contained cytochrome P-450, PBRLM-5, DPLC, cytochrome P-450 reductase and NADPH-generating system. The sample cuvette also contained halothane. (B) Spectrum obtained when cytochrome b_5 was added to both cuvettes in addition to the complete mixture. Preparations were analyzed at time intervals of 2 min following the addition of halothane. Key: (a) before halothane addition; (b) 2 min, (c) 4 min, (d) 6 min, and (e) 8 min following halothane addition.

DISCUSSION

Previous studies from this laboratory have offered evidence that halothane is reduced by two electrons; the first transferred from the heme moiety can be accomplished by other reduced heme proteins or by heme and, therefore, is not a specific cytochrome P-450 function, and the second electron transfer is specific to cytochrome P-450 and very likely is a property requiring the protein moiety [16, 28]. The first electron transfer to halothane results in the production of CTE and also the lipid-bound product, the formation of which reportedly involves a free radical. The second electron transfer results in the formation of CDE. It is likely that the two-electron transfer takes place sequentially while halothane is associated with the cytochrome. The release of the free radical ($CF_3\dot{C}ClH$) and the subsequent covalent binding of product to lipids, or the formation of CTE, may result from an interruption in the sequence because of a slow second electron transfer.

It is clear from the data presented in this paper that halothane is reduced to CDE by purified PBRLM5, that the reduced halothane–cytochrome P-450 complex forms with PBRLM5, and that cytochrome b_5 apparently interacts with this cytochrome P-450 form to enhance both the complex formation and subsequent CDE production. Previous studies have shown that the production of CDE and the formation of the halothane–cytochrome P-450 complex which absorbs at 470 nm are very low when measured in intact hepatic microsomes from untreated rats. Both measurements increase dramatically in microsomes from phenobarbital-treated animals [18]. The studies reported herein are consistent with the previous results since the PBRLM5 form is the predominant form found in phenobarbital-induced microsomes [4] and may be in very low levels in untreated hepatic microsomes. It should be noted that the purified forms of cytochrome P-450 used in this study produced lower amounts of CDE per unit of cytochrome P-450 than we have found previously in microsomes [18]. Since these cytochrome P-450 preparations contained a relatively high amount of glycerol (25%), primarily to assure stability during storage, we assume this may have contributed to the decreased enzymatic activity. However, it should be noted that the presence of glycerol enhances the complex formation of halothane and reduced cytochrome P-450 possibly by stabilizing the complex in a manner similar to that of hexane ([18] and unpublished observation). Therefore, the absorption at 470 nm reflects the optimum formation of the complex and, since the CDE is derived from the complex under these circumstances, the absorption at 470 nm is a valid indicator of enzymatic activity.

The stimulation of the complex formation and CDE production by cytochrome b_5 may be the result of (1) the stabilization of the halothane–cytochrome P-450 complex so that the second electron may be transferred more readily or (2) a facilitation by cytochrome b_5 of the input of the second electron [29]. The fact that PBRLM4 (RLM5a) was not active in producing CDE, although it produced large amounts of CTE whether cytochrome b_5 was present or not, is noteworthy, particularly since PBRLM4 also did not form a spectral complex with cytochrome b_5. RLM2, which showed some activity in producing CDE, although no halothane–RLM2 complex was detected, appeared to be inhibited by cytochrome b_5 in transferring the second electron to halothane.

A number of investigators have shown that addition of purified cytochrome b_5 to the reconstituted monooxygenase system can have an inhibitory or stimulatory effect on metabolism depending on the cytochrome P-450 isozyme involved and the substrate utilized [25, 30–36]. The stimulatory effect of cytochrome b_5 in the reconstituted system has been attributed to a direct electron transfer from cytochrome b_5 to cytochrome P-450 and/or interaction between the two proteins [24, 29, 30, 33, 34, 37–39]. The data presented in this study do not explain the mechanism of how cytochrome b_5 interacts with cytochrome P-450 but the evidence suggests that the enhancement of cytochrome P-450 activity by b_5 is not uniform for all forms of cytochrome P-450 and leaves open the possibility, as proposed by Tamburini *et al.* [29], that there is a specificity for the interaction of cytochrome b_5 with cytochrome P-450 isozymes.

The data presented in this report show that at least two forms of cytochrome P-450, RLM2 and PBRLM5, are capable of catalyzing the two-electron reduction of halothane to CDE, at approximately equal rates. However, cytochrome b_5, which unlike other hemes and hemoproteins, is incapable itself of catalyzing a one-electron reduction of halothane [28], and other halocarbons [40–42] had a differential effect upon the two-electron transfer process: while more than doubling CDE production via PBRLM5, cytochrome b_5 virtually blocked all production of CDE by RLM2, without a corresponding compensatory elevation of CTE. These results lead us to conclude that, as with other forms of cytochrome P-450, the influence of cytochrome b_5 is determined both by the form of cytochrome P-450 and the substrates utilized [43].

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