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ISOFLURANE AND CYTOCHROME b_5 STIMULATION OF 2-CHLORO-1,1-DIFLUOROETHENE METABOLISM BY RECONSTITUTED RAT CYP2B1 AND CYP2C6

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Abstract—Isoflurane stimulates the metabolism of 2-chloro-1,1-difluoroethene (CDE) in liver microsomes from phenobarbital-treated rats or rabbits. The P450 isozymes involved and the mechanism by which such stimulation occurs have not been clarified. The present study examined the effects of isoflurane and cytochrome b_5 on CDE metabolism in reconstituted systems containing purified rat CYP2B1 or CYP2C6. Under similar incubation conditions, CYP2B1 defluorinated CDE at approximately five times the rate of CYP2C6. Isoflurane was a potent stimulator of CDE metabolism, increasing it nearly 5-fold when catalyzed by CYP2B1, but only 2-fold when catalyzed by CYP2C6. Isoflurane had no stimulatory effect on benzphetamine metabolism by CYP2B1 or CYP2C6. Cytochrome b_5 was not required for isoflurane-facilitated CDE metabolism; however, the addition of cytochrome b_5 to CYP2B1 increased CDE metabolism 71 and 44%, in the absence and presence of isoflurane, respectively. In reconstituted CYP2B1, isoflurane generated a type I difference spectrum of approximately twice the magnitude of CDE and stimulated NADPH consumption more so than CDE. The same quantity of NADPH was consumed when CDE was present with isoflurane as compared with isoflurane alone. These data support the hypothesis that isoflurane stimulates CDE metabolism by a mechanism involving increased P450 reduction via direct isoflurane interaction with P450.

Key words: isoflurane; chlorodifluoroethene; metabolism; stimulation; cytochrome b_5; CYP2B1

The ability of one P450 substrate to directly facilitate, rather than inhibit, the metabolism of another has been reported for only a limited number of compound mixtures [1–4]. Such interactions are rare due to the fact that for facilitation to occur, the stimulatory compound must not effectively inhibit the substrate from accessing the P450 active site, and it must, to a greater degree than the substrate, facilitate or render more efficient some aspect of the P450 process that leads toward substrate oxidation.

The ability of the volatile fluorinated anesthetics and related compounds to directly increase the oxidation of a number of haloethenes *in vivo* and in liver microsomes represents a novel type of facilitatory interaction that appears to occur between gaseous haloethenes and short-chain saturated halocarbons. For example, in rat liver microsomes, halogenated ethenes whose metabolism is increased by the halocarbons are: vinyl chloride [5], trifluoroethene [6], and the halothane metabolite CDE† [7, 8]. Compounds that facilitate the metabolism of these haloethenes are: halothane ($CF_3CBrClH$), TCE, isoflurane ($CF_3CClHOCF_2H$), and several chlorofluorocarbon substitutes including HCFC-123 (CF_3CCl_2H) [9]. These interactions result from the activities of specific P450 forms, since increased haloethene metabolism occurs to a low degree or not at all in microsomes from untreated, or β-naphthoflavone- or isoniazid-treated rats, but is very pronounced (up to a 3.5-fold increase) in animals treated with phenobarbital [5, 6].

Studies of the model interactive compounds isoflurane and CDE showed that they contrast substantially in the manner in which they interact with P450 in rabbit microsomes [10]. At equal concentrations, isoflurane induced approximately twice the NADPH and oxygen consumption as did CDE. Further, the isoflurane-induced P450 activity was mostly uncoupled from substrate metabolism, whereas the lesser CDE-induced activity was highly coupled to CDE defluorination. When isoflurane and CDE were present together, the consumption of NADPH and oxygen was equal to that found when isoflurane alone was present, even though CDE metabolism was approximately tripled. This suggested that isoflurane-stimulated P450 activity supports the oxidation of CDE at greater rates [10].

The halocarbon interactions in microsomes that lead to enhanced haloethene metabolism are unknown; likewise, whether similar effects occur in reconstituted P450 systems has not been determined. Isoflurane and CDE may interact directly with one or more components of the P450 enzyme system to increase CDE defluorination, or isoflurane may interact at a site apart from the P450 protein. Other microsomal proteins, such as cytochrome b_5, are stimulatory to some P450 activities [11] and may be activated by isoflurane. To clarify the processes by which the stimulatory interactions occur, the present study investigated the effects of isoflurane and cytochrome b_5 on CDE defluorination in recon-

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† Abbreviations: CDE, 2-chloro-1,1-difluoroethene; TCE, 1,1,1-trichloroethane; and SOD, superoxide dismutase.

stituted P450 systems containing CYP2B1 and CYP2C6, two major phenobarbital-inducible isozymes in the rat.

MATERIALS AND METHODS

Chemicals. Isoflurane (Forane) was obtained from Ohmeda, Inc. (Liberty Corner, NJ) and 2-chloro-1,1-difluoroethene (> 99%) from PCR Chemicals, Inc. (Gainesville, FL). Catalase (58,000 U/mg), SOD (3800 U/mg), hydroquinone, and horseradish peroxidase (275 U/mg, type 12) were purchased from Aldrich–Sigma Chemicals (St. Louis, MO).

Animals. Male Sprague–Dawley rats, 180–200 g, were purchased from Sasco, Inc., and housed in accordance with the National Institutes of Health guidelines for animal care. The rats were treated by i.p. injection with 80 mg phenobarbital/kg body weight for 5 days. On day 5, animals were killed by the inhalation of carbon dioxide, and liver microsomes were prepared by differential centrifugation. Microsomes were either used immediately for enzyme purification, or stored concentrated at −70° in 0.1 M $NaPO_4$, 1 mM EDTA buffer (pH 7.4) until used for microsomal assays. For cytochrome b_5 purification, nontreated rats were anesthetized with halothane and decapitated. The livers were removed, perfused with 1.1% KCl, and stored at −70° until used.

Cytochrome P450 purification. CYP2B1 and CYP2C6 were purified using the chromatography scheme of Backes *et al.* [12] with *n*-octylamino-Sepharose 4B as prepared by Guengerich and Martin [13]. The P450 fraction was eluted from *n*-octylamino-Sepharose 4B using a buffer (pH 7.25) containing 0.1 M potassium phosphate, 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.33% sodium cholate, and 0.08% Emulgen 911. This P450 fraction was applied to hydroxyapatite, and the cytochrome P450 eluted with 90 mM KPO_4 was collected. After dialysis in 5 mM KPO_4, the P450 fraction was applied to DEAE-Sephacel. The CYP2C6-containing fraction did not adsorb and was eluted with 5 mM KPO_4. The fraction enriched in CYP2B1 was eluted with 35 mM KPO_4 buffer. CYP2B1 and CYP2C6 were purified further by chromatography on hydroxyapatite using 10–100 mM KPO_4 gradients. The molecular weights of CYP2B1 and CYP2C6 were calculated as 53,300 and 52,400, respectively, following slab-gel electrophoresis of the proteins [14]. The CYP2B1 protein had a specific content of 13.8 nmol P450/mg protein and the CYP2C6 protein, 7.2 nmol/mg protein. The identity of CYP2B1 was further confirmed as CYP2B1 by western-blot analysis using a polyclonal rabbit anti-rat CYP2B1 antibody supplied by Dr. James Halpert.

Cytochrome P450 reductase. NADPH–cytochrome P450 reductase was eluted from the *n*-octylamino-Sepharose 4B column using a buffer (pH 7.25) containing 0.1 M potassium phosphate, 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2 μM flavine mononucleotide, 0.1 mM phenylmethylsulfonyl fluoride, 0.35% sodium cholate, and 0.15% sodium deoxycholate. NADPH–cytochrome P450 reductase was purified further as previously described [13] with the exception that 2′,5′-ADP Sepharose 4B (Pharmacia) was substituted for 2′,5′-ADP agarose. The purified NADPH–cytochrome P450 reductase reduced cytochrome *c* at a rate of 40,000–48,000 nmol cytochrome *c*/mg protein.

Cytochrome b_5 *purification.* Cytochrome b_5 was purified by a modification of the procedure of Spatz and Strittmatter [15]. Livers from nontreated rats were homogenized in 3 vol. of 1.1% KCl, and microsomes were prepared. The microsomal pellets were resuspended in 100 mM Tris-acetate, 1 mM EDTA (pH 8.1) and sonicated for 2 min following the addition of NaCl to give 1 M. The suspensions were centrifuged at 108,000 *g* for 1 hr, and the microsomal pellets were resuspended in 100 mM Tris-acetate, 1 mM EDTA (pH 8.1). The latter step was repeated.

The microsomes were then treated with acetone and extracted with Triton X-100; cytochrome b_5 was initially fractionated on DEAE-Sephacel (substituted for DEAE-cellulose) [15]. Fractions containing cytochrome b_5 were combined, concentrated to 2–4 mL using a Nucleopore ultrafiltration device with an Amicon PM-10 membrane, and applied to a Sephadex G-100 column equilibrated with 10 mM Tris-acetate (pH 8.1) containing 0.1 mM EDTA [16]. This step was repeated. Fractions containing cytochrome b_5 were combined and concentrated to 2–4 mL. The cytochrome b_5 preparation was diluted to 50 mL with 50 mM $NaPO_4$ (pH 7.25) and reconcentrated to 2–4 mL. Cytochrome b_5 dilution and reconcentration were repeated, and the cytochrome b_5 was reapplied to a Sephadex G-100 column equilibrated with 50 mM $NaPO_4$ (pH 7.25) for final chromatography. Following elution, the cytochrome b_5 fraction was dialyzed overnight in 100 vol. of 50 mM $NaPO_4$ (pH 7.25). The final cytochrome b_5 preparation had a specific content of 45.7 nmol cytochrome b_5/mg protein.

Reconstituted enzyme systems. Cytochrome P450 (CYP2B1 or CYP2C6) and NADPH–cytochrome P450 reductase were added to a solution of 8 mM L-α-dilauroylphosphatidylcholine [17]. The final molar ratios of P450:reductase:dilauroylphosphatidylcholine were 1:1.2:165. For enzyme systems containing cytochrome b_5, cytochrome b_5 was added in a 1:1 molar ratio to P450. This mixture was diluted to the desired P450 concentration with 50 mM $NaPO_4$ (pH 7.25) and allowed to stand at room temperature for 2 hr prior to incubation. Incubations with CDE, benzphetamine, and/or isoflurane contained 0.5 μmol NADPH, 5 μmol glucose-6-phosphate and 0.33 U glucose-6-phosphate dehydrogenase in 0.5 mL, and were contained in 6.27 mL plastic hypovials sealed with Hycar septa (Pierce Chemical Co., Rockford, IL). All CDE or isoflurane additions are indicated as percent (v/v) in air.

Microsomal incubations. One-milliliter incubations contained 3 mg microsomal protein, 2.5 μmol glucose-6-phosphate, 0.125 U glucose-6-phosphate dehydrogenase, and 0.5 μmol NADPH or 0.5 μmol NADH, or both. Incubations were performed in sealed 6.27 mL plastic hypovials.

NADPH consumption. Measurements of NADPH consumption were performed spectrophotometrically at 30° using a Cary 3E UV/visible spectro-

Table 1. Effects of isoflurane (IF) and 1,1,1-TCE (TCE) on CDE defluorination and the effects of isoflurane on benzphetamine (Benz) demethylation by rat CYP2B1 in the absence and presence of cytochrome b_5

Treatment	CDE defluorination (nmol fluoride/nmol CYP2B1)	Treatment	Benzphetamine demethylation (nmol formaldehyde/nmol CYP2B1)
IF	2.1 ± 0.4		
CDE	9.7 ± 0.6	Benz	168 ± 0
CDE + b_5	25.3 ± 2.7*	Benz + b_5	152 ± 5†
CDE + IF	45.8 ± 1.3*	Benz + IF	171 ± 8
CDE + IF + b_5	58.9 ± 3.0‡	Benz + IF + b_5	137 ± 5†§‖
CDE + TCE	50.6 ± 0.7*		
CDE + TCE + b_5	67.2 ± 1.4‡¶		

Incubations of CDE contained 0.83% CDE, TCE, or isoflurane. Benzphetamine incubations (1 mM) contained 0.83% isoflurane in the headspace where noted. Incubations were for 10 min at 37° and contained 0.6 nmol P450/mL. P450 was reconstituted as described in Materials and Methods. Values are the means ± SEM of triplicate measurements.

* Significant from CDE ($P < 0.05$).
† Significant from benz ($P < 0.05$).
‡ Significant from CDE + b_5 ($P < 0.05$).
§ Significant from benz + b_5 ($P < 0.05$).
‖ Significant from benz + IF ($P < 0.05$).
¶ Significant from CDE + TCE ($P < 0.05$).

photometer equipped with a Cary temperature controller. Microsomes, reconstituted P450 systems, or P450-reductase–cytochrome *c* suspensions (0.6 mL) were sealed in 1.4 mL quartz cuvettes into which two tubes were inserted. One tube (Clay Adams PE20) was for the introduction of the gases and NADPH, and the other (Clay Adams PE50) for pressure equalization. Gas mixtures (5 mL) containing CDE, isoflurane, or CDE plus isoflurane, in air were bubbled through the reaction mixture with a gas-tight syringe. The preparations were allowed to stand for 3 min, and then 0.2 mM NADPH was introduced, followed immediately by the addition of another 0.5 mL of the appropriate gas mixture. The absorbance at 340 nm was followed for 1–2 min. NADPH consumption was calculated using an NADPH extinction coefficient of 6.2 $mM^{-1}\,cm^{-1}$ at 340 nm [18].

The effects of CDE and isoflurane on cytochrome *c* reduction by NADPH reductase as measured by NADPH oxidation were determined as described by Strobel and Dignam [19] with the exception that measurements were made in a 50 mM $NaPO_4$ buffer (pH 7.25). Reaction mixtures (0.7 mL) contained 1.6 pmol cytochrome P450 reductase, 28 nmol cytochrome *c* and 260 pmol L-α-dilauroylphosphatidylcholine. Cytochrome *c* reduction was determined following the addition of 0.1 mM NADPH.

Binding spectra of isoflurane and CDE with CYP2B1. Type I difference binding spectra were measured in sealed 1.4 mL quartz cuvettes at 25°. Samples were scanned from 350 to 500 nm using a Cary 3E UV/visible spectrophotometer. Gases were introduced as described above. Maximum difference spectra were attained in 2 min.

Assays. Protein was assayed by the method of Lowry *et al.* [20]. Fluoride was assayed with fluoride selective electrodes [21], and formaldehyde was measured by the method of Nash [22]. Cytochrome P450 was assayed by the CO reduced difference spectra [23]. NADPH–cytochrome P450 reductase activity was determined at 25° as described by Strobel and Dignam [19] using a reduced cytochrome *c* extinction coefficient of 21 $mM^{-1}\,cm^{-1}$ at 550 nm. NADPH–cytochrome P450 reductase and cytochrome b_5 were quantified as described by Tamburini *et al.* [16].

Statistics. One-way ANOVA with the Scheffe's F-test was used to test for significance. P values < 0.05 were considered significant.

RESULTS

The reconstituted system containing CYP2B1 effectively metabolized CDE as determined by fluoride release (Table 1). In accord with the resistance of isoflurane to metabolism, isoflurane was poorly metabolized by CYP2B1. Under similar 10-min incubations (0.83%), only 2.1 nmol fluoride/nmol CYP2B1 was formed from isoflurane. The addition of isoflurane to CDE-containing incubations increased defluorination approximately 5-fold. In identical incubations containing benzphetamine as substrate, isoflurane had no significant effect on benzphetamine demethylation. Confirmation that increased fluoride release was due to CDE metabolism is that the effect of TCE on defluorination was similar to that of isoflurane. TCE, however, was slightly more effective in increasing fluoride release which is likely due to its greater lipid solubility, a factor determined to play a role in haloethane-facilitated CDE metabolism [9].

The addition of cytochrome b_5 to the reconstituted CYP2B1 system increased CDE metabolism, but decreased benzphetamine demethylation (Table 1). Cytochrome b_5 increased CDE metabolism 160% in the absence of isoflurane, and further facilitated CDE metabolism 28% in the presence of isoflurane. Consequently, the CYP2B1 system that most effectively metabolized CDE was one that contained

Table 2. Effects of NADH on CDE metabolism in liver microsomes from phenobarbital-treated rats in the absence and presence of isoflurane (IF)

	CDE defluorination (nmol fluoride/mg protein)		
	IF	CDE	CDE + IF
NADPH	ND	12.8 ± 0.5	26.0 ± 2.6*
NADH	ND	1.3 ± 0.2†	2.9 ± 0.7†
NADPH + NADH	ND	16.0 ± 0.2†‡	34.6 ± 2.2*‡

Incubations were performed as described in Materials and Methods and were for 10 min at 37°. Vials contained CDE (0.91%), IF (0.46%), or CDE (0.91%) plus IF (0.46%). ND indicates non-detectable. Values are means ± SEM of triplicate measurements.

* Significant from the corresponding CDE group ($P < 0.05$).

† Significant from the corresponding NADPH group ($P < 0.05$).

‡ Significant from the corresponding NADH group ($P < 0.05$).

Table 3. NADPH consumption during CDE metabolism by CYP2B1 in the absence and presence of isoflurane (IF)

	NADPH (nmol consumed/nmol CYP2B1)	Fluoride (nmol produced/nmol CYP2B1)
Control	35.8 ± 3.7	
IF	78.3 ± 3.1*	1.8 ± 0.3
CDE	45.6 ± 4.0†	23.2 ± 0.4†
CDE + IF	81.8 ± 3.6*‡	68.1 ± 10.2†‡

NADPH consumption was measured as described in Materials and Methods and in the legend of Fig. 1. NADPH consumption was quantitated at 1 min and fluoride at 6 min. Values are means ± SEM of triplicate measurements.

* Significant from the control group ($P < 0.05$).

† Significant from the IF group ($P < 0.05$).

‡ Significant from the CDE group ($P < 0.05$).

both isoflurane and cytochrome b_5, which produced a defluorination rate of six times greater than when the system did not contain these additions. Evidence of an electron transfer role of cytochrome b_5 in CDE metabolism in liver microsomes is that the addition of NADH to NADPH-containing incubations further increased CDE metabolism 25 and 33% in the absence and presence of isoflurane, respectively (Table 2). NADH alone poorly supported CDE metabolism as compared with NADPH.

NADPH consumption in reconstituted CYP2B1 systems containing isoflurane, CDE, or CDE plus isoflurane is shown in Table 3. CDE weakly stimulated CYP2B1 activity as measured by NADPH oxidation at 1 min of incubation. Isoflurane increased NADPH consumption 2.1 times over that observed with CDE alone (7.3 times minus control). In these preparations, the combination of CDE and isoflurane produced the identical degree of NADPH consumption as did isoflurane alone. The same degree of NADPH consumption by isoflurane, and isoflurane plus CDE, was similarly observed over time (Fig. 1). Measurement of fluoride in these incubations at 6 min showed an increase in CDE defluorination of 3-fold (Table 3). The lesser degree of CDE metabolism, and stimulation in these assays was due to the fact that NADPH was by necessity limiting to the reaction.

The type I binding spectra observed when CDE and isoflurane interact with CYP2B1 are depicted in Fig. 2. At equal concentrations, CDE produced a binding spectrum of lesser magnitude than isoflurane. Isoflurane generated a greater binding spectrum of which the absorbance difference at 380–420 nm was similar in magnitude to the spectrum when isoflurane and CDE were added.

Neither isoflurane nor CDE was found to have any effect on the ability of purified cytochrome P450 reductase to reduce cytochrome *c*. Values determined (μmol cytochrome *c* reduced/mg protein/min ± SEM) were: control (no addition), 14.15 ± 0.81; isoflurane (2% in air), 14.43 ± 1.05; CDE (2%), 14.58 ± 0.26; and CDE + isoflurane (2% each), 13.48 ± 0.397.

To determine whether P450-dependent hydroxyl radical formation or other reduced oxygen forms may be responsible for CDE metabolism, CDE defluorination by CYP2B1 was analyzed in the presence of catalase and/or SOD, hydroquinone and horseradish peroxidase. Incubations were carried out as described in Table 1 and contained the following additives where noted: catalase, 1000 U/mL; SOD, 0.2 mg/mL; peroxidase, 50 U/0.4 mL; hydroquinone, 0.2 mM. No addition inhibited CDE defluorination. Values obtained (nmol F^-/nmol P450/10 min ± SEM) were: CDE, 9.7 ± 0.6; CDE + catalase, 8.1 ± 0.2; CDE + SOD, 8.7 ± 0.1; and CDE + SOD + catalase, 9.3 + 0.1. Values obtained in the presence of isoflurane were: CDE + isoflurane, 45.8 ± 1.3; CDE + isoflurane + catalase, 47.4 ± 0.7; CDE + isoflurane + SOD, 45.4 ± 0.9; and CDE + isoflurane + SOD + catalase, 53.7 ± 0.6. In another set of experiments, the effects of horseradish peroxidase and hydroquinone were determined. Neither had an effect on CDE defluorination in either the absence or presence of isoflurane. Values obtained (nmol F^-/nmol P450/10 min ± SEM) were: CDE, 13.1 ± 0.2; CDE + peroxidase, 14.5 ± 0.4; CDE + hydroquinone, 12.0 ± 0.2; CDE + isoflurane, 65.5 ± 1.1; CDE + isoflurane + peroxidase, 69.7 ± 3.3; and CDE + isoflurane + hydroquinone, 67.4 ± 1.5.

The reconstituted system containing CYP2C6 metabolized CDE at a rate of less than 25% that of CYP2B1 (Table 4). Furthermore, the addition of isoflurane increased defluorination by only 118% in the absence or presence of cytochrome b_5. As occurred with CYP2B1, cytochrome b_5 facilitated the metabolism of CDE whether or not isoflurane was present. The defluorination of isoflurane by CYP2C6 could not be detected. As opposed to the inhibitory effects of cytochrome b_5 on the metabolism of benzphetamine by CYP2B1 as observed here and elsewhere [11], cytochrome b_5 facilitated the metabolism of this substrate by CYP2C6, which is likewise in accord with previous results [24].

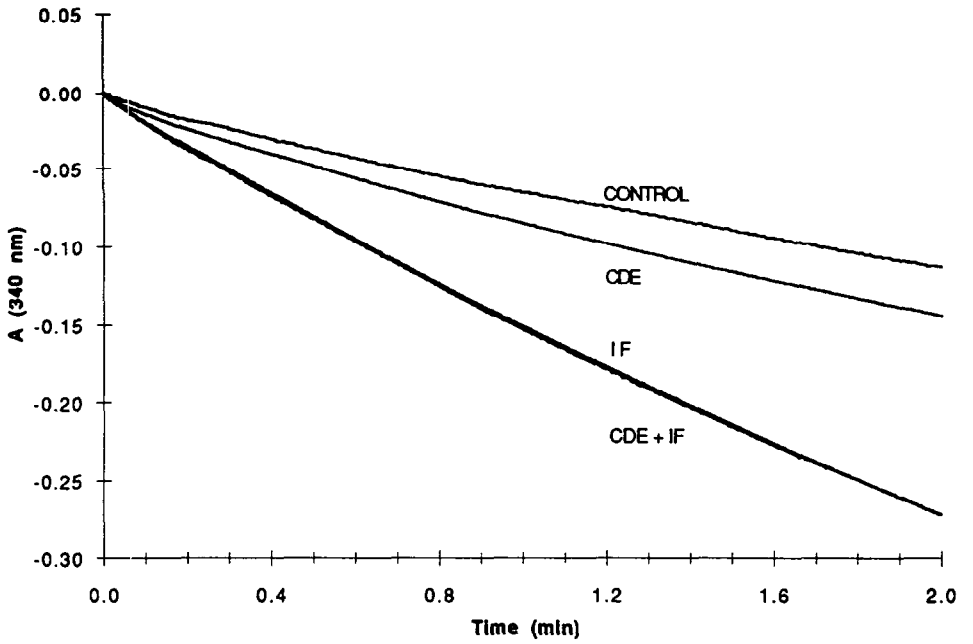

Fig. 1. Time-course for the oxidation of NADPH in reconstituted CYP2B1 systems containing 2% CDE, 2% isoflurane (IF), 2% CDE plus 2% isoflurane (CDE + IF), or no addition (control). Reaction mixtures contained 0.3 nmol/mL CYP2B1 in 50 mM $NaPO_4$ buffer (pH 7.25) and were performed as described in Materials and Methods. Absorbance measurements were made every 0.27 sec (signaling averaging time of 0.067 sec). Each curve was constructed from the average of each time point from three runs. (The IF and CDE + IF curves are superimposable.)

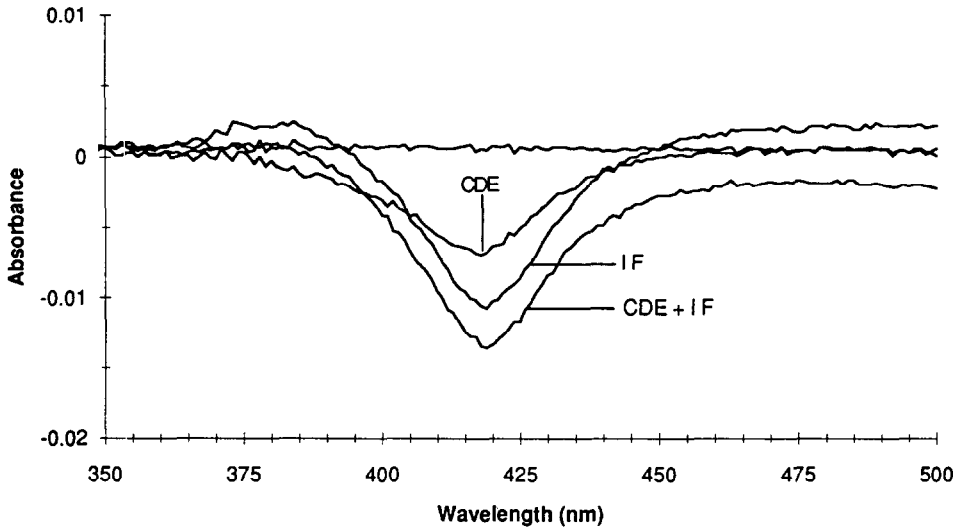

Fig. 2. CYP2B1 difference spectra following the addition of CDE, isoflurane, or CDE plus isoflurane at 2% each. Measurements were made as described in Materials and Methods. Cuvettes contained 1.5 nmol/mL CYP2B1 and 300 nmol/mL L-α-dilauroylphosphatidylcholine in 50 mM $NaPO_4$ buffer (pH 7.25).

DISCUSSION

The present study demonstrates that isoflurane as well as TCE can increase the defluorination of CDE in reconstituted P450 systems containing either CYP2B1 or CYP2C6. These data confirm that CDE metabolism is dependent on P450 activity, that cytochrome b_5 plays no role in isoflurane-facilitated CDE metabolism, and that isoflurane and CDE directly interact with one or more of the essential components of the P450 system to increase CDE defluorination.

Table 4. Effects of isoflurane (IF) on CDE defluorination and benzphetamine (Benz) demethylation by rat CYP2C6 in the absence and presence of cytochrome b_5

Treatment	CDE defluorination (nmol fluoride/nmol CYP2C6)	Treatment	Benzphetamine demethylation (nmol formaldehyde/nmol CYP2C6)
IF	ND		
IF + b_5	ND		
CDE	1.94	Benz	147 ± 6.2
CDE + b_5	3.84	Benz + b_5	199 ± 2.3*
CDE + IF	4.22	Benz + IF	126 ± 2.3*†
CDE + IF + b_5	9.22	Benz + IF + b_5	172 ± 3.5*†‡

Methods are as described for Table 1 except that benzphetamine incubations contained 0.3 nmol P450/mL. CDE values represent the mean of duplicate measurements, and benzphetamine demethylation values are the means ± SEM of triplicate determinations. ND indicates non-detectable.

* Significant from benz ($P < 0.05$).

† Significant from benz + b_5 ($P < 0.05$).

‡ Significant from benz + IF ($P < 0.05$).

In cases where facilitated metabolism has been studied previously, it has not been determined unambiguously how the process of stimulation occurs. Increased electron transfer, however, has been implicated. Huang *et al.* [1] postulated that 7,8-benzoflavone activated benzo[*a*]pyrene hydroxylation in rat and guinea pig liver microsomes by increasing the flow of electrons between P450 reductase and P450. Miller *et al.* [2] hypothesized that betamethazone stimulated the 2-hydroxylation of biphenyl in rat liver microsomes by enhancing the interaction of reductase with P450. Less clear in regard to electron flow is the mechanism of Wolff *et al.* [3], who proposed that the ability of ethanol and acetone to facilitate aldrin epoxidation in reconstituted CYP2B1 and CYP2B2 preparations resulted from binding of the solvents to a site on P450 apart from the type I and II binding sites.

When the effects of CDE and isoflurane on P450 are contrasted, it is clear that isoflurane acts to increase P450 reduction, and does so by binding to the type I site [25, 26]. This is shown by an increase in NADPH consumption and the greater isoflurane-induced type I difference spectrum. As a consequence of increased P450 reduction, there is an increase in the rate of formation of reduced oxygen species [25], which in the case of isoflurane exposure alone is predominately uncoupled activity because of the resistance of isoflurane to metabolism [27].

Because no more NADPH is utilized when isoflurane and CDE are added together, it is apparent that the P450 activity occurring when isoflurane is present in the CYP2B1 preparation (non-isoflurane-dependent plus isoflurane-dependent activity) is in some manner utilized to support CDE metabolism. Previous studies confirmed the above relationship since equal oxygen was consumed in rabbit microsomes in the presence of isoflurane, or isoflurane plus CDE, where CDE defluorination was more than tripled [10]. CDE cannot effectively displace the much more lipid-soluble isoflurane from the type I site, or from other lipophilic sites at near equivalent concentrations; therefore, increased CDE metabolism occurred while isoflurane was bound to the type I site. The higher lipid solubility of isoflurane is shown by the fact that isoflurane has an olive oil : gas partition coefficient of 98 [28], whereas that determined for CDE in this laboratory is 13.

Greater CDE metabolism in the presence of isoflurane-stimulated P450 activity could possibly occur via an increase in release of reduced oxygen species, such as the reactive hydroxyl radical, which hydroxylates aniline for example [29]; or CDE may gain access to the P450-bound active oxygen where active oxygen is generated at greater rates. Evidence that CDE is metabolized at the P450 heme-iron, as are most P450 substrates, is that scavengers of superoxide anion, hydrogen peroxide, and hydroxyl radicals did not affect CDE metabolism in either the absence or presence of isoflurane. Likewise, a secondary peroxide driven mechanism for CDE metabolism is ruled out by a lack of effect of peroxidase on CDE defluorination.

Although CDE and isoflurane are expected to compete in binding to the P450 type I site, unhindered CDE metabolism could occur by the fact that isoflurane may not exclude CDE from interacting with the P450 heme-iron. Isoflurane and CDE could bind in a cooperative fashion, or CDE may access the heme-iron in a direct manner without being substantially impeded by isoflurane. Small molecules, such as CO and O_2, for example, freely interact with the P450 heme-iron regardless of whether any compound is bound, presumably by approaching the active site from the aqueous phase. Although larger than CO or O_2, CDE, as well as vinyl chloride and trifluoroethene, has higher probabilities of gaining access to sites restricted in size compared with the vast majority of P450 substrates. It can be speculated that CDE accesses the P450 peroxy heme of isoflurane-bound P450, and that a process equivalent to metabolic switching occurs where, at the point of P450 branching, the activated oxygen species generated by P450 attacks CDE rather than being released as reduced oxygen species forming hydrogen peroxide and/or water [30, 31]. Evidence that haloethene size is critical to the stimulatory process is that the defluorination of a larger gaseous olefin,

hexafluoropropene, was not stimulated, but inhibited by isoflurane in microsomes from phenobarbital-treated rats (data not shown).

It cannot be ruled out from the present data that isoflurane in some manner acts in part to increase the binding affinity or binding orientation of CDE in the P450 active sites. Nor can it be excluded that isoflurane binding to a site apart from the type I site plays a role in increased CDE metabolism. Clarification of these mechanisms by kinetic analyses is hampered by the large degree of CDE mechanism-based P450 inactivation [7]. The ability of cytochrome b_5 to stimulate CDE defluorination apart from the action of isoflurane is likely due to its ability to contribute the second electron to the substrate–oxycytochrome P450 complex, as proposed for its facilitatory role in the metabolism of other substrates [32].

In conclusion, this study demonstrates that isoflurane and TCE can potentiate CDE metabolism by each compound directly interacting with components of the P450 enzyme system. Furthermore, because the degree of stimulation was greater in reconstituted CYP2B1 than in rat liver microsomes [7] or in reconstituted CYP2C6, it is apparent that the phenobarbital-inducible P450, CYP2B1 [33], makes a major contribution to the stimulatory interaction observed in phenobarbital-treated rats.

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