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FLUROXENE (2,2,2-TRIFLUOROETHYL VINYL ETHER) MEDIATED DESTRUCTION OF CYTOCHROME P-450 *IN VITRO**

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Abstract—Incubation of rat hepatic microsomes with an NADPH generating system and the anaesthetic fluroxene (2,2,2-trifluoroethyl vinyl ether) at 30° resulted in the destruction of hepatic cytochrome P-450 in excess of that observed in the presence of the NADPH generating system alone. The extent of destruction of cytochrome P-450 was markedly enhanced by pre-induction of cytochrome P-450 with phenobarbital. Induction with 3-methylcholanthrene or 3,4-benzpyrene, which induce cytochrome P-448, enhanced the overall level of cytochrome destruction. No destruction was observed when CO was added to the system prior to the addition of the anaesthetic, or when NADH replaced NADPH or when fluroxene was replaced by its chemically reduced form, 2,2,2-trifluoroethyl ethyl ether. The fluroxene potentiated destruction of cytochrome P-450 was accompanied by the loss of heme from the microsomes but not by the appearance of cytochrome P-420 nor by the loss of cytochrome b_5 or NADPH–cytochrome *c* reductase. We conclude that cytochrome P-450 is specifically destroyed by fluroxene in a metabolic process involving destruction of its heme group. The vinyl group of the anaesthetic is essential for the destructive process.

A heterogenous class of hepatic microsomal drug metabolizing enzymes known collectively as cytochrome P-450 catalyses the detoxification of an extremely wide variety of xenobiotics[1]. This group of enzymes normally functions to metabolize various hydrophobic compounds, converting them to relatively more hydrophilic products which are then more readily excreted by the body[1]. Although many substances induce the biosynthesis of cytochrome P-450[2], relatively few compounds are known specifically to degrade hepatic microsomal cytochrome P-450 *in vivo* or *in vitro*. The latter compounds include the extremely hazardous 2-allyl-2-*iso* propylacetamide (AIA) which produces experimental porphyria in animals[3, 4], allyl containing barbiturates[5] and the lethal toxins CS_2, CCl_4 and dimethylnitrosoamine[6–9]. We have, however, recently reported on the destruction of hepatic microsomal cytochrome P-450 *in vivo* following anaesthesia of rats with fluoroxene (2,2,2-trifluoroethyl vinyl ether), a widely used, normally non-toxic fluorocarbon anaesthetic agent.‡ We describe here our investigations into the destruction of hepatic microsomal cytochrome P-450 *in vitro* by this anaesthetic. These studies were performed with a view to gaining insight into the mechanism of the destruction of cytochrome P-450 *in vivo*.

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MATERIALS AND METHODS

Sodium phenobarbital (PB) and 3-methylcholanthrene (MC) were obtained from Maybaker, S. A. and Eastman-Kodak, respectively. 3,4-benzypyrene (BP) was from Sigma Chemicals. Water was glass distilled and deionized. 2-Allyl-2-*iso* propylacetamide was a generous gift of Hoffman-La Roche, Nutley, NJ. NADP and glucose-6-phosphate dehydrogenase were purchased from Miles Laboratories. NADH was from Nutritional Biochem. Corp. Fluroxene (2,2,2-trifluoroethyl vinyl ether) was supplied by Ohio-Medical Products, Madison, WI. 2,2,2-Trifluoroethyl ethyl ether (TFEE) was prepared by hydrogenation of fluroxene at 30 lb/in^2 hydrogen using 5 g activated palladium on carbon catalyst (Merck) per 250 ml fluroxene. Hydrogenation was continued until samples of reaction-mixture exhibited no further absorbance at 1600 cm^{-1} in the infrared spectrum, characteristic of carbon–carbon double bonds. The reduced fluroxene was refluxed for at least 24 hr over sodium and was subsequently purified by fractional distillation. The fraction boiling between 50·0 and 50·4° was collected and stored in the dark under an atmosphere of nitrogen. The TFEE was tested for peroxides with 5% aqueous KI (w/v) immediately before use. Male rats of the Wistar strain weighing 180–230 g were used in all experiments. Induction of type P-450 cytochromes was by intraperitoneal injection of sodium phenobarbital (80 mg/kg per day in 0·9% sterile saline), 3,4-benzpyrene (40 mg/kg per day in corn oil) or 3-methylcholanthrene (40 mg/kg per day in corn oil) for 3 consecutive days. Microsomes were prepared from fresh rat liver homogenates by gel filtration on Sepharose 2B in

0·15 M KCl–0·02 M Tris–HCl, pH 7·4, according to the method of Tangen *et al.*[10], and were used immediately after elution from the column.

Microsomes (2·0 mg protein/ml) were incubated and shaken at 30° in the presence or absence of an NADPH generating system. In some experiments 30 mM fluroxene or TFEE or 5 mM AIA were added. When CO was used in controls it was bubbled through the reaction mixture for one minute prior to the addition of fluroxene. When the NADPH generating system was utilized, the incubation mixture of 12·0–12·6 ml microsomal suspension contained 4·0 μmoles NADP, 90·0 μmoles glucose-6-phosphate, 3 U. glucose-6-phosphate dehydrogenase, 6·4 μmoles $MgCl_2$ and 12 μmoles nicotinamide. Additional NADP was required at 25 min (3 μmoles) and 50 min (2 μmoles) after the commencement of the reaction. In one experiment with phenobarbital-induced microsomes, 20 μmoles NADH replaced the NADP and NADPH generating system. Boosters of 3 μmoles NADH were added at 25 and 50 min. The concentrations of type P-450 cytochromes were determined as described by Omura and Sato[11] at 0, 10, 30 and 60 min after the initiation of the reaction. All assays of cytochrome P-450 are accurate to ±0·03 nmole/mg protein.

Microsomal heme was determined as the pyridine hemochromogen using the method of Omura and Sato[11]. Cytochrome b_5 content and NADPH–cytochrome *c* reductase activity were measured at 0 and 30 min[11, 12]. Spectral studies on microsomal suspensions were performed in a Unicam SP 1800 recording spectrophotometer in a compartment adjacent to the photomultiplier.

RESULTS

The effects of fluroxene and TFEE on the concentrations of type P-450 cytochromes in hepatic microsomes isolated from uninduced, 3,4-benzpyrene, 3-methylcholanthrene and phenobarbital induced rats are presented in Figs. 1–4. The term type P-450 cytochromes will be used to refer to mixtures of cytochrome P-450 and cytochrome P-448. Incubation of hepatic microsomes from uninduced or induced rats in the absence or presence of fluroxene, TFEE or AIA did not produce appreciable alterations in microsomal type P-450 cytochromes concentration (Figs. 1–4). Addition of the NADPH generating system alone, produced a small decrease in cytochrome P-450 content of microsomes from uninduced rats (Fig. 1)—larger quantities of type P-450 cytochromes were degraded from microsomes of induced rats which had initially elevated levels of these cytochromes (Fig. 4). The destruction of type P-450 cytochromes following addition of TFEE and NADPH to microsomes was in all cases not increased relative to that seen with NADPH alone (Figs. 1–4), but incubation of microsomes in the presence of fluroxene (same concentration as that of TFEE) and NADPH, resulted in destruction of type P-450 cytochromes in excess of that seen with NADPH alone (Fig. 1). No similar destruction of cytochrome P-450 was noted when NADH was used in place of NADPH. This decrease in cytochrome P-450 content produced by fluroxene

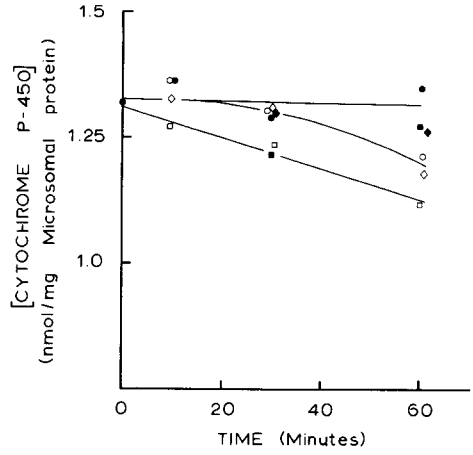

Fig. 1. The destruction of hepatic microsomal cytochrome P-450 from uninduced rats. Microsomal cytochrome P-450 concentrations in the presence of: (●), no additive; (■), fluroxene (30 mM); (◆), TFEE (30 mM); (○), NADPH generating system; (□), NADPH generating system + fluroxene (30 mM); (◇), NADPH generating system + TFEE. Microsome concentration (2·0 mg protein/ml), experiments performed at 30°.

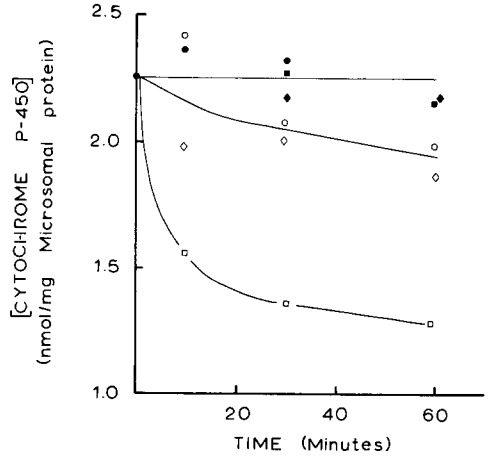

Fig. 2. The destruction of hepatic microsomal cytochrome P-450 from rats induced with 3,4-benzpyrene (40 mg/kg per day for 3 days). Microsomal cytochrome P-450 concentrations in the presence of: (●), no additive; (■), fluroxene (30 mM); (◆), TFEE (30 mM); (○), NADPH generating system; (□), NADPH generating system + fluroxene (30 mM); (◇), NADPH generating system + TFEE. Microsome concentration (2·0 mg protein/ml), experiments performed at 30°.

and NADPH was very slight in control microsomes, but was marked in microsomes with initially elevated levels of cytochrome P-450 (c.f. Fig. 1 and Fig. 4). The destruction of cytochrome P-450 in phenobarbital induced microsomes following the addition of fluroxene was prevented by bubbling CO through the reaction mixture prior to the addition of the anaesthetic. The loss of cytochrome P-450 from microsomes induced with phenobarbital is in agreement with the decrease in heme content of the microsomes determined as pyridine hemochromogen. In one experiment the ratio of cytochrome P-450 concentrations before the addition of fluroxene to that 1 hr after addition was 1·4 and the

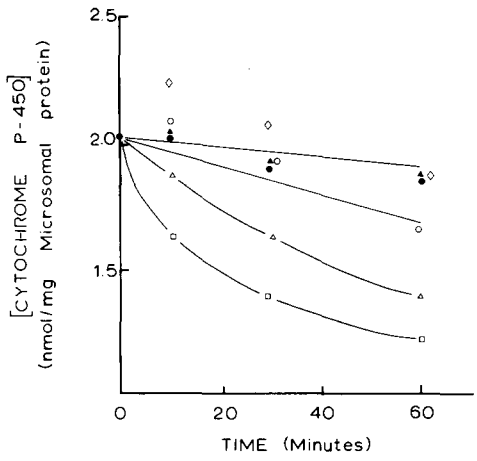

Fig. 3. The destruction of hepatic microsomal cytochrome P-450 from rats induced with 3-methylcholanthrene (40 mg/kg per day for 3 days). Microsomal cytochrome P-450 concentrations in the presence of: (●), no additive; (▲) AIA (5 mM); (○), NADPH generating system; (□), NADPH generating system + fluroxene (30 mM); (◇), NADPH generating system + TFEE; (△), NADPH generating system + AIA (5 mM). Microsome concentration (2·0 mg protein/ml), experiments performed at 30°.

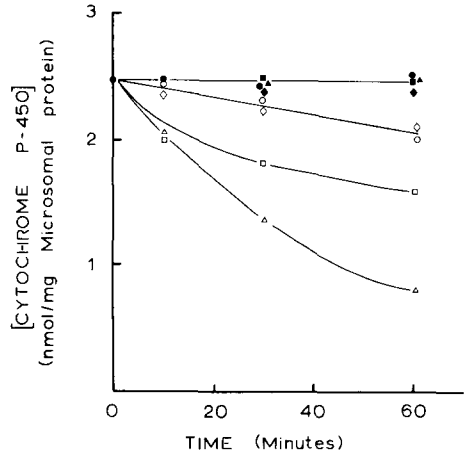

Fig. 4. The destruction of hepatic microsomal cytochrome P-450 from rats induced with phenobarbital (80 mg/kg per day for 3 days). Microsomal cytochrome P-450 concentrations in the presence of: (●), no additive; (■), fluroxene (30 mM); (◆), TFEE (30 mM); (▲), AIA (5 mM); (○), NADPH generating system; (□), NADPH generating system + fluroxene (30 mM); (◇), NADPH generating system + TFEE (30 mM); (△), NADPH generating system + AIA (5 mM). Microsome concentration (2·0 mg protein/ml), experiments performed at 30°.

corresponding ratio of heme was 1·5. The effect of AIA, a compound known to specifically destroy cytochrome P-450 *in vivo* and *in vitro* [3, 4, 13, 14], on hepatic microsomal cytochrome P-450 content is included for comparison (Figs. 3 and 4). As has been previously reported, AIA rapidly destroys cytochrome P-450 in phenobarbital induced microsomes, in the presence of NADPH (Fig. 4)[3]. Further, as we have confirmed (Fig. 3), AIA is unable to destroy cytochrome P-448, the predominant type P-450 cytochrome present in microsomes from 3-methylcholanthrene induced rats, or at least affects this hemoprotein to a markedly lesser extent than the cytochrome P-450 which predominates in phenobarbital induced animals[3].

The effects of the addition of fluroxene to phenobarbital induced microsomes on the levels of microsomal cytochrome b_5 and NADPH cytochrome *c* reductase are presented in Table 1. It is clear that while levels of the NADPH–cytochrome *c* reductase are diminished after the addition of the NADPH generating system for 30 min no additional decreases are observed following introduction of fluroxene to the system. No breakdown of cytochrome b_5 was observed after the addition of fluroxene to microsomes containing the NADPH generating system.

DISCUSSION

From previous investigations of the destruction of cytochrome P-450 *in vivo* and *in vitro* by AIA[7] or by allyl containing barbiturates[5, 15] it is clear that a number of factors must be satisfied for destruction to occur: the compound mediating the destruction must contain an allyl group[5, 7, 15], in the case of AIA it binds to cytochrome P-450[16] and is required to be metabolized[7], and a mechanism involving lipid peroxides is not operative[15]. It was also concluded that the diminished levels of cytochrome P-450 produced by AIA were not a consequence of inhibition of synthesis but rather of increased rates of degradation of cytochrome P-450[5].

The results of the present investigation reveal that similar conclusions can be drawn regarding the fluroxene mediated destruction of cytochrome P-450 *in vitro*. Comparison of the relative effects of fluroxene and TFEE in inducing the destruction of microsomal cytochrome P-450 depicted in Figs. 1–4 demonstrates that the chemical reduction of the vinyl group in synthesising TFEE from fluroxene effectively overcomes the destructive effect of fluroxene. Clearly then the vinyl group of fluroxene is an essential component for the destruction of cytochrome P-450. The inability of TFEE to produce diminished cytochrome P-450 levels cannot be attributed to its lack of binding to the heme protein since we have demonstrated* that TFEE binds almost as firmly to cytochrome P-450 as fluroxene does. Apparently the vinyl ether group of fluroxene is equivalent to an allyl group in its ability to act as a source of metabolite capable of producing the destruction of cytochrome P-450.

A number of experimental results indicate that the *in vitro* destruction of microsomal cytochrome P-450 mediated by fluroxene requires prior, cytochrome P-450 catalysed, metabolism of the anaesthetic. These include the prevention of the fluroxene mediated destruction by carbon monoxide which is a well established inhibitor of cytochrome P-450[17] and the specific requirement for NADPH as the electron donor before destruction of cytochrome P-450 occurs. The enhanced levels of destruction of cytochrome P-450 observed with microsomes having initially elevated levels of

* K. M. Ivanetich, J. J. Bradshaw, J. A. Marsh and L. S. Kaminsky, in preparation.

Table 1. Effects of fluroxene (30 mM) on phenobarbital induced hepatic microsomal cytochrome b_5 and NADPH–cytochrome *c* reductase concentrations *in vitro*

Reagents added	Time after addition of reagents (min)	Concn cytochrome b_5 (nmoles/mg protein)	Concn NADPH–cytochrome *c* reductase (units/mg protein)
NADPH generating system	0	0·57	0·30
NADPH generating system	30	0·56	0·21
NADPH generating system + fluroxene	0	0·53	0·29
NADPH generating system + fluroxene	30	0·54	0·21

Reagents were added to microsomal suspensions (2 mg protein/ml).

cytochrome P-450, as a result of induction by phenobarbital, is further evidence that it is a metabolite of fluroxene which is responsible for the destructive effects observed. On the other hand the fluroxene mediated destruction observed with 3-methylcholanthrene (Fig. 3) and 3,4-benzpyrene (Fig. 2) induced hepatic microsomes is less readily explained. The observation that fluroxene forms a spectrally detectable complex with cytochrome P-450, but not with cytochrome P-448 suggests that fluroxene does not bind to cytochrome P-488*. It is therefore unlikely that fluroxene would specifically destroy this hemoprotein. In addition, the *in vivo** studies demonstrate a much greater loss of the cytochrome from animals with elevated cytochrome P-450 than with elevated cytochrome P-448 content. The amount of cytochromes P-450 destroyed under conditions of 3-methylcholanthrene or 3,4-benzpyrene induction is small enough that the results could be accounted for by the destruction of cytochrome P-450 alone, but not cytochrome P-448 alone.

In view of the failure of fluroxene to affect the concommitant destruction of other components of the microsomal electron transport system, while mediating the destruction of cytochrome P-450, it is possible that destruction occurs as the metabolite is formed at the active site of the cytochrome. Since there is a corresponding loss of microsomal heme as the levels of cytochrome P-450 are diminished, without any appearance of cytochrome P-420, it can be concluded that the destruction of cytochrome P-450 involves the degradation of its heme moiety rather than a conformational rearrangement of the protein. The reactive metabolite must thus interact with the heme of cytochrome P-450 at a rate greater than the rate of dissociation of the ES complex. It is, however, possible that the reactive metabolite reacts with and destroys cytochrome P-450 specifically.

It has previously been suggested[7] that destruction of cytochrome P-450, of the type observed in the present investigation, could arise from the cytochrome P-450 catalysed epoxidation of the allyl group in AIA. The possibility that a similar epoxidation of the vinyl group of fluroxene is responsible for the observed degradation of the heme group of cytochrome P-450 is being actively investigated in our laboratories.

From the studies reported herein it would be anticipated that many other compounds containing unsaturated carbon–carbon bonds may also catalyze the destruction of cytochrome P-450 *in vivo* and *in vitro*. This destruction would be expected to be maximal in animals (or man) pretreated with inducing agents for cytochrome P-450 such as the barbiturates. The resultant effects from the destruction of cytochrome P-450 *in vivo* are open to speculation.

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