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THE DEGRADATION OF DIFFERENT FORMS OF CYTOCHROME P-450 IN VIVO BY FLUROXENE AND ALLYL-ISO-PROPYLACETAMIDE

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SUMMARY: Treatment of uninduced, phenobarbital and 3-methylcholanthrene induced rats with fluroxene and allyl-iso-propylacetamide decreased hepatic microsomal cytochrome P-450 and equivalently decreased microsomal heme, aniline binding and p-nitroanisole demethylase. In contrast, ethylmorphine demethylase, benzpyrene-3-hydroxylase and ethoxyresofurin deethylase were not in all cases decreased in proportion to the loss of cytochrome P-450. After phenobarbital induction fluroxene and allyl-iso-propylacetamide degrade multiple forms of cytochrome P-450, but degrade in the greatest amounts the form(s) of cytochrome P-450 inducible by phenobarbital. After 3-methylcholanthrene induction fluroxene preferentially degrades cytochrome P-448, while allyl-iso-propylacetamide is relatively specific for the form(s) of cytochrome P-450 inducible by phenobarbital.

The volatile anesthetic agent fluroxene (2,2,2-trifluoroethyl vinyl ether) and the porphyrogenic compound allyl-iso-propylacetamide (AIA)* are known to degrade the heme of cytochrome P-450 *in vivo* and *in vitro* (1-4). Both compounds appear to require prior metabolism by microsomal enzymes in order to degrade cytochrome P-450; the activating enzyme in the case of fluroxene appears to be cytochrome P-450 (1-5). Both fluroxene[+] and AIA (1) convert the heme of cytochrome P-450 to unidentified green pigments (6).

Through the use of inducing agents and enzyme assays we have attempted to ascertain the effect of modification of the heme of cytochrome P-450 by fluroxene and AIA on enzyme activity and to clarify which, if any, of the multiple forms of cytochrome P-450 are preferentially degraded by fluroxene and AIA. Aniline binding and p-nitroanisole demethylation were considered to be characteristic of multiple forms of cytochrome P-450 (7,8). Benz-

* Abbreviations used are AIA, allyl-iso-propylacetamide; MC, 3-methylcholanthrene; PB, phenobarbital.

[+] J.J. Bradshaw and K.M. Ivanetich, unpublished observations.

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pyrene 3-hydroxylation and ethoxyresofurin deethylation were thought to be characteristic primarily of the polycyclic hydrocarbon inducible forms of cytochrome P-450 known as cytochrome P-448, whilst ethylmorphine demethylation was considered to be characteristic primarily of the form(s) of cytochrome P-450 inducible by phenobarbital (9-12).

EXPERIMENTAL: Materials were obtained as follows: p-nitroanisole, Eastman Kodak; 3,4-benzpyrene, Koch-Light. 3-hydroxybenzpyrene was a gift from Dr Harry Gelboin, N.I.H. Allyl-iso-propylacetamide was a gift from Hoffman-La Roche. Ethyl isocyanide was prepared by the method of Jackson and McKusick (13). Ethoxyresofurin was prepared from resofurin (Eastman-Kodak) as described by Burke and Mayer (14).

Groups of 6 to 8 male Wistar rats (180+5 g) were induced with PB or MC as described earlier (4). The animals were starved overnight after the last injection and the following morning some of the animals received fluroxene intraperitoneally or AIA subcutaneously. After PB induction, the rats received 2 ml/kg fluroxene or 200 mg/kg AIA and were killed 55+5 min thereafter. After MC induction, the rats received 5 ml/kg fluroxene and were killed 85+5 min after the fluroxene injection, or received 200 mg/kg AIA and were killed 55+5 min thereafter. Hepatic microsomes were prepared as described earlier (4) and were used for all assays at a concentration of 2 mg microsomal protein/ml 0.02 M Tris-HCl, pH 7.4, unless otherwise indicated. The maximal binding of aniline to hepatic microsomal cytochrome P-450 ($A_{430} - A_{395}$) was measured in the presence of 87 mM aniline. The ethyl isocyanide crossover pH was measured with 1.5 mg microsomal protein/ml 0.1 M potassium phosphate buffer and 2.8 mM ethyl isocyanide (15). Benzpyrene-3-hydroxylase activity was measured at 25° as described by Prough et al. (16). p-Nitroanisole demethylase was measured as per Netter and Seidel (8) using 1.3 mg microsomal protein/ml. Hepatic microsomal glucose-6-phosphatase, ethylmorphine demethylase and ethoxyresofurin deethylase were measured as described in the literature (14,17,18). All other experimental details were as described earlier (3,4). Reported values are means + standard deviations for determinations on two to four different preparations of microsomes. Student's t test was utilized for statistical analysis, with statistical significance being taken as $p < .01$.

RESULTS: The ability of fluroxene and AIA to degrade cytochrome P-450 hemoproteins in vivo under the conditions of our experiments is confirmed in Table 1. Each type of treatment shown in Table 1 resulted in equivalent losses of hepatic microsomal heme and cytochrome P-450, with the losses of heme and cytochrome P-450 being the greatest following PB and MC induction for fluroxene and PB induction for AIA. The concentrations of the other microsomal enzymes measured were unaffected by fluroxene ($p > .05 - .5$) (Table 1).

Table 1. The effects of fluroxene and AIA *in vivo* on the levels of hepatic microsomal proteins and heme[a]

Induction	Additional treatment	Cyt P-450[b]	Heme[b]	Loss heme[b]/ Loss cyt P-450[b]	Cyt b_5[b]	NADPH-cyt c reductase (u/mg microsomal protein)	Glucose-6-phosphatase (mg P_i/mg microsomal protein/20 min)
PB	None	2.26±.26	2.80±.12	-	.670±.059	.135±.019	.109±.014
	Fluroxene	1.34±.38	1.94±.39	.86/.92	.616±.078	.121±.021	.106±.020
	AIA	0.68±.13	1.22±.23	1.58/1.58			
MC	None	1.44±.06	2.54±.30	-	.688±.056	.089±.013	.135±.026
	Fluroxene	0.67±.03	1.68±.05	.86/.77	.648±.074	.087±.022	.127±.017
	AIA	1.19±.05	2.01±.23	.53/.25			
None	None	0.99±.04	1.74±.09	-			
	Fluroxene	0.61±.01	1.45±.05	.29/.38			
	AIA	0.54±.01	1.22±.04	.52/.45			

[a] Abbreviation used is cyt, cytochrome. [b] In nmol/mg microsomal protein.

Following fluroxene and AIA treatment, hepatic microsomal aniline binding and p-nitroanisole demethylase were decreased in proportion to the decrease in the levels of cytochrome P-450 hemoproteins for all types of induction (Table 2). Following fluroxene treatment of PB induced rats, all other activities were decreased in proportion to the loss of cytochrome P-450, except for the absence of any effect on the levels of benzpyrene hydroxylase. In MC induced rats none of the remaining activities were decreased by fluroxene in proportion to the losses of cytochrome P-450. Following AIA treatment of PB induced rats, all activities were decreased in proportion to the loss of cytochrome P-450, whereas AIA treatment of MC induced rats did not significantly decrease benzpyrene hydroxylase ($p > .25$) or ethoxyresofurin deethylase ($p = .025$) but did disproportionately decrease ethylmorphine demethylase. The ethyl isocyanide crossover pH of PB induced microsomes ($7.57 \pm .08$) was not altered by fluroxene or AIA. In MC induced microsomes, the crossover pH ($6.98 \pm .02$) was unaffected by AIA, but was increased to $7.56 \pm .10$ following fluroxene treatment.

DISCUSSION: The lack of effect of fluroxene (Table 1) and AIA (1,2) on the levels of hepatic microsomal cytochrome b_5, NADPH-cytochrome c reductase and glucose-6-phosphatase would appear to indicate that these compounds do not affect microsomal components non-specifically in contrast to CCl_4 which degrades glucose-6-phosphatase in addition to cytochrome P-450 (19,20). In as much as the losses of hepatic microsomal heme, aniline binding and p-nitroanisole demethylase were equivalent to the losses of cytochrome P-450 under all conditions (Tables 1 and 2), it is evident that fluroxene and AIA decrease enzyme activity in proportion to the loss of heme of cytochrome P-450, and it would appear that there may be a causal relationship between the loss of heme and the loss of enzyme activity.

The sensitivity of different forms of cytochrome P-450 to fluroxene and AIA appears to be altered by prior induction by PB or MC. In PB in-

Table 2. The effects of fluroxene and AIA on hepatic microsomal cytochrome P-450 levels and activities in uninduced and induced rats.

Induction	Additional treatment	Cyt P-450	Aniline Binding	p-NO_2 Anisole Demethylase	Ethylmorphine Demethylase	Benzpyrene-3-Hydroxylase	Ethoxyresofurin Deethylase
PB	Fluroxene	63±13	64±3	68±1	73±2	107±3[b]	49±14
MC	Fluroxene	47±7	40±11	48±15	72±12[a]	37±2[a]	13±12[b]
-	Fluroxene	62±3	62±2	N.D.	N.D.	N.D.	N.D.
PB	AIA	32±8	23±3	30±11	22±5	33±8	31±10
MC	AIA	80±5	73±3	86±11	51±5[b]	92±9[a]	87±4
-	AIA	55±4	47±5	N.D.	N.D.	N.D.	N.D.

Abbreviations used are cyt, cytochrome; N.D., not determined. Reported values are percentages of activity remaining after fluroxene or AIA treatment. In the absence of fluroxene or AIA treatment, aniline binding (absorbance) was .21, .19, and .10 in PB, MC induced and uninduced microsomes, respectively; other activities (nmol/min/mg microsomal protein) in PB and MC induced rats were: p-nitroanisole demethylase 7.3, 5.4; ethylmorphine demethylase 1.05, 0.37; benzpyrene-3-hydroxylase 0.17, 0.41; ethoxyresofurin deethylase 0.21, 17.8.

[a] $p < .01$ relative to loss of cytochrome P-450. [b] $p < .001$ relative to loss of cytochrome P-450.

duced rats, fluroxene appears to be non-specific for multiple forms of cytochrome P-450 except that the form(s) of cytochrome P-448 which catalyse(s) benzpyrene-3-hydroxylation was (were) not affected. In MC induced rats, fluroxene appears to specifically degrade cytochrome P-448 in as much as: [1] there was a disproportionately slight loss of ethylmorphine demethylase and disproportionately great losses of benzpyrene hydroxylase and ethoxyresofurin deethylase and [2] the ethyl isocyanide crossover pH was increased from 6.98, which is characteristic of cytochrome P-448, to 7.56, the pH which is characteristic of the form(s) of cytochrome P-450 induced by phenobarbital and is also found for uninduced microsomes (11,15). Following both types of induction, fluroxene decreased ethoxyresofurin deethylase to a greater extent than benzpyrene hydroxylase ($p < .0005$) suggesting that, although both benzpyrene hydroxylase and ethoxyresofurin deethylase are inducible by MC, fluroxene differentially affects the forms of cytochrome P-450 catalyzing the two reactions‡. Quantitatively, the majority of the cytochrome P-450 hemoprotein molecules degraded by fluroxene after PB induction would appear to be the form(s) of cytochrome P-450 induced by PB[x], whereas after MC induction, the form(s) of cytochrome P-448 induced by MC were degraded in the greatest amounts by fluroxene.

AIA appears to degrade multiple forms of cytochrome P-450 non-specifically in PB induced rats. After MC induction AIA would appear to preferentially degrade the form(s) of cytochrome P-450 induced by PB while not significantly decreasing cytochrome P-448 (Table 2). The lack of effect of AIA on the ethyl isocyanide crossover pH is consistent with the proposed effects of AIA on the different forms of cytochrome P-450 in MC and PB

‡ The differential induction by MC of benzpyrene-3-hydroxylase (3 fold) and of ethoxyresofurin deethylase (ca. 150 fold) (9) suggests that different forms of MC inducible cytochrome P-450 (21) may catalyze the two reactions.

x In as much as these are the predominant forms of cytochrome P-450 after phenobarbital induction (23).

induced rats. Quantitatively, the form(s) of cytochrome P-450 inducible by PB would appear to be degraded to the greatest extent by AIA after either PB or MC induction. This proposal is consistent with the observation of Levin et al. (2) that following PB induction AIA causes greater loss of cytochrome(s) P-450 of short half life in CO binding particles in vitro.

The observed differences in the specificities of fluroxene and AIA for different forms of cytochrome P-450 may reflect differences in the enzymes catalyzing the metabolic activation of the two compounds or differences in the susceptibilities of the multiple forms of cytochrome P-450 to the reactive metabolites of fluroxene and AIA. Activities associated with different forms of cytochrome P-450 were also not decreased in proportion to the losses of cytochrome P-450 mediated by vinyl chloride in vitro (22). The differential effects of PB and MC induction on the degradation of cytochrome P-450 hemoproteins by fluroxene and AIA and also by vinyl chloride (22) could reflect the considerations mentioned above but could also reflect the differential effects of these inducing agents on detoxifying enzymes such as epoxide hydrase.

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