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Involvement of Cytochrome P-450c in α-Naphthoflavone Metabolism by Rat Liver Microsomes

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SUMMARY

Metabolism of α-naphthoflavone (ANF) is increased markedly in rat liver microsomes by 3-methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), two inducers of cytochromes P-450c and P-450d (P-450c and P-450d). Although several indirect lines of evidence in the literature suggest that ANF is metabolized by P-450c, Vyas *et al.* [*J. Biol. Chem.* **258**:5649–5659 (1983)] reported that ANF metabolism by 3-MC-induced rat liver microsomes was only partially inhibited by antibodies against P-450c. Our laboratory has previously reported clastogenic effects of metabolites of ANF, and in the present study we reexamined the role of P-450c in ANF metabolism by both uninduced and TCDD-induced rat liver microsomes, using monospecific polyclonal antibodies to P-450c and P-450d. ANF metabolism was inhibited to different extents in TCDD-induced microsomes by different preparations of anti-P-450c. One lot of anti-P-450c produced only 50% inhibition of ANF metabolism in TCDD-induced microsomes, whereas another lot of anti-P-450c inhibited ANF metabolism by 80%. Anti-P-450d had no effect on ANF metabolism. Neither anti-P-450c nor anti-P-450d inhibited ANF metabolism in uninduced rat liver microsomes. In a reconstituted enzyme system, purified P-450c metabolized ANF 47 and 510 times more rapidly than P-450d and P-450b, respectively. Metabolites resulting from oxidation at 7,8- or 5,6-positions (7,8-dihydro-7,8-dihydroxy-ANF, 5,6-dihydro-5,6-dihydroxy-ANF, 5,6-oxide-ANF, and 6-hydroxy-ANF) were formed by all preparations of microsomes. An unknown toxic ANF metabolite was formed only with a reconstituted P-450c system and with 3-MC- or TCDD-induced microsomes. Our results indicate that P-450c is responsible for the majority of the metabolism of ANF in TCDD-induced microsomes, whereas other constitutive isozymes are responsible for the metabolism seen in uninduced liver microsomes. The variable inhibition of ANF metabolism with different lots of anti-P-450c probably reflects the differences in the proportion of antibodies to different epitopes important in the binding or metabolism of this substrate.

ANF is known as a specific *in vitro* inhibitor of P-450c, and it also modifies the carcinogenic actions of certain polycyclic aromatic hydrocarbons, e.g., BP (1). ANF is metabolized by the microsomal monooxygenase system (2–6), and there is evidence that certain ANF metabolites are more potent inhibitors of P-450c than ANF (7–8). Our laboratory has shown that ANF induces sister chromatid exchanges in lymphocytes from human populations exposed to cigarette smoke or polychlorinated biphenyls and dibenzofurans (9–10), when added to these cells *in vitro*. ANF also induces large amounts of sister chromatid exchanges and chromosome aberrations in Chinese hamster ovary cells when coincubated with liver microsomes from TCDD-induced rats, but no such changes are detected with liver microsomes from phenobarbital-induced or uninduced rats (11). The clastogenic activation of ANF by TCDD-induced microsomes is NADPH dependent, which suggests an involvement of P-450 isozymes (11). Cigarette smoke components (e.g., BP), polychlorinated biphenyls, dibenzofurans, and TCDD are all known to interact with a cellular receptor system (*Ah* receptor) that leads to a marked induction of P-450c and P-450d in liver microsomes of rats (12), whereas phenobarbital induces a different cytochrome, P-450b (13).

We recently demonstrated that ANF metabolism is induced markedly by TCDD but affected only slightly by phenobarbital (11, 14). These results are consistent with those of previous studies (2–6), which report that 3-MC, β-naphthoflavone, and Arochlor-1254, all inducers of P-450c and P-450d, increase ANF metabolism. Taken together, these findings indicate an important role for P-450c or P-450d in ANF metabolism. However, surprisingly, Vyas *et al.* (6) reported that ANF metabolism by 3-MC-induced rat liver microsomes was inhibited only slightly by antibodies against purified P-450c and was not inhibited by antibodies raised against either P-450d or P-450a. Although ANF was metabolized by purified P-450c in a recon-

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ABBREVIATIONS: ANF, α-naphthoflavone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3-MC, 3-methylcholanthrene; 7,8-dihydrodiol, 7,8-dihydro-7,8-dihydroxy-ANF; 5,6-dihydrodiol, 5,6-dihydro-5,6-dihydroxy-ANF; 9,10-dihydrodiol, 9,10-dihydro-9,10-dihydroxy-ANF; BSA, bovine serum albumin; EH, epoxide hydrolase; P-450, cytochrome P-450; HPLC, high pressure liquid chromatography; BP, benzo[*a*]pyrene.

stituted enzyme system, these authors concluded that uncharacterized P-450 isozymes may be more important than P-450c in the metabolism of ANF by 3-MC-induced rat liver microsomes.

Because of our interest in the increased production of clastogenic ANF metabolites by TCDD-induced microsomes and in human lymphocytes from smokers and humans exposed to polychlorinated biphenyls, we reinvestigated the role of P-450c in ANF metabolism in the present study. We examined the effects of monospecific polyclonal antibodies to purified P-450c and P-450d on ANF metabolism in uninduced and TCDD-induced rat liver microsomes. We also examined metabolism of ANF by reconstituted enzyme systems containing different purified P-450s. The present studies provide strong evidence that P-450c is the primary isozyme involved in metabolic activation of ANF by TCDD-induced rat liver microsomes.

Materials and Methods

Chemicals and radiochemicals. [^{3}H]ANF (specific activity, 16.5 Ci/mmol) was synthesized by Chemsyn Science Laboratories (Lenexa, KS). The method of preparation guaranteed that the label was in the 4′-position of the phenyl ring. The end product was purified by thin layer chromatography. The chemical and radiochemical purity, checked by HPLC, were greater than 99%. Hydroxy-ANF standards were a gift of Dr. S. Nesnow (Environmental Protection Agency, Research Triangle Park, NC). TCDD was obtained from Dr. J. McKinney (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The purity, determined by HPLC, was 99%.

Microsomes. 3-MC-induced liver microsomes were prepared from male Sprague-Dawley rats (80–150 g) as previously described (14). Liver microsomes were prepared by differential centrifugation from female Sprague-Dawley rats (180–220 g, 9–11 weeks old; Charles River Breeding Laboratory, Wilmington, MA) 7 days after a single oral dose of TCDD (10 μg/kg of body weight) in corn oil and from uninduced rats.

Antibodies and purified enzymes. Antiserum against P-450c (anti-P-450c) was raised in goat (lot 1) or rabbit (lot 2), and antiserum against P-450d (anti-P-450d) was raised in goat, as previously described (15). IgG was prepared by ammonium sulfate precipitation and DEAE-cellulose chromatography, as described by Thomas *et al.* (16). Nonadsorbed polyclonal antibodies to purified P-450c and P-450d inherently cross-react with the heterologous hemoprotein, because of the immunochemical similarity of these cytochromes (15, 16). Monospecific antibodies were prepared by absorption of anti-P-450c against P-450d and of anti-P-450d against P-450c, using the appropriate antigen covalently bound to Sepharose 4B (17). Cross-reactivity to the heterologous antigen was <3% for anti-P-450d and <1% for anti-P-450c, as analyzed by Western blots. Lot 2 anti-P-450c was also adsorbed against control male and female rat liver microsomes, to remove minor cross-reactivity to lower molecular weight peptide bands that presumably are constitutive P-450s. Western blots using anti-P-450d antibodies recognized only one polypeptide band in control or TCDD-induced rat liver microsomes, corresponding in molecular weight to P-450d. Western blots with lot 2 anti-P-450c recognized only one band in TCDD-induced microsomes and no bands in control microsomes.

P-450c, P-450d, and P-450b were isolated from 3-MC-3,4,5,3′,4′5′-hexachlorobiphenyl-, and phenobarbital-treated rats, respectively (15). NADPH-cytochrome *c* reductase was purified by the method of Yasukochi and Masters (18), as modified by Serabjit-Singh *et al.* (19). One unit is defined as reducing 1 nmol of beef heart cytochrome *c*/min at 25°, in 0.3 M phosphate buffer, pH 7.7. EH was a generous gift from Dr. Andrew Parkinson (University of Kansas Medical Center).

Antibody inhibition of microsomal ANF metabolism. Microsomes and varying concentrations of antibody (range, 10–100 mg of IgG/mg of microsomal protein) were preincubated at room temperature for 5 min before addition of 10 μM [^{3}H]ANF (specific activity, 5 Ci/mmol) and an NADPH-generating system (0.3 mM NADP, 16 mM isocitrate, 0.3 units/ml isocitrate dehydrogenase; Sigma), in 50 mM potassium phosphate buffer (pH 7.5) containing 5 mM $MgCl_2$ and 0.5% BSA. Preimmune rabbit or goat IgGs were added as necessary to maintain the same total concentration of IgG in each sample. After 20 min, the incubations were terminated by addition of 5 volumes of water-saturated ethyl acetate.

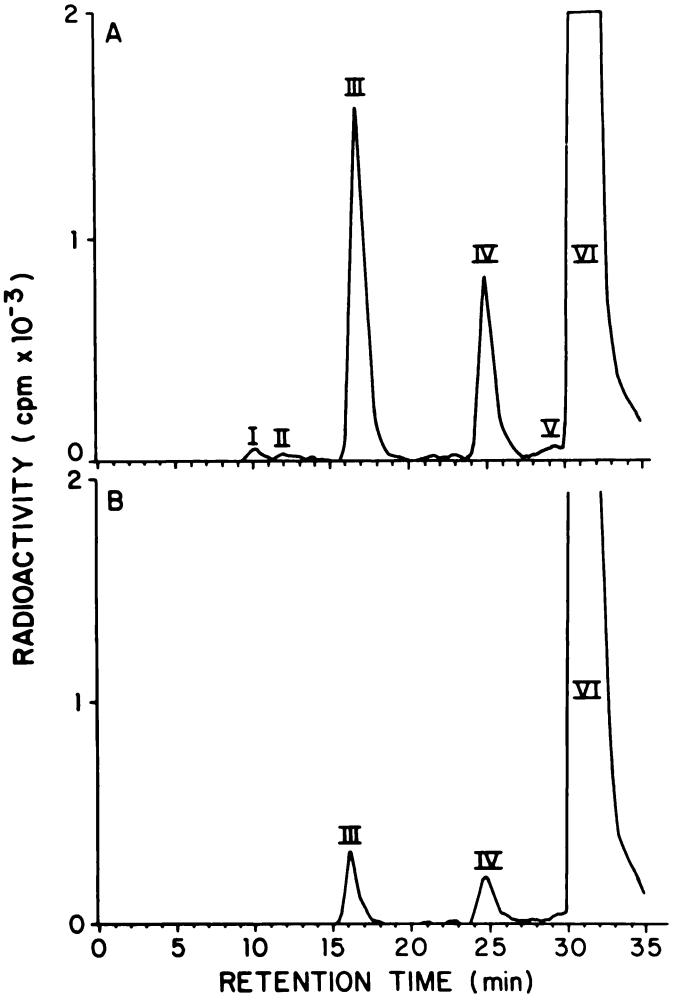

Fig. 1. HPLC chromatograph of ANF metabolites formed in the absence or presence of rabbit anti-P-450c. A, 0.02 mg/ml TCDD-induced microsomes were preincubated with preimmune rabbit IgG. [^{3}H]ANF (10 μM) was added and the reaction was carried out for 20 min at 37°. B, 0.02 mg/ml TCDD-induced microsomes were preincubated with anti-P-450c (100 mg/mg of microsomal protein). [^{3}H]ANF (10 μM) was added and the reaction was carried out for 20 min at 37°. The peaks are 9,10-dihydrodiol (*I*), 5,6-dihydrodiol (*II*), 7,8-dihydrodiol (*III*), 5,6-oxide-ANF (*IV*), 6-hydroxy-ANF (*V*), and ANF (*VI*). Minor metabolites eluting between peaks III and IV are unknown. Following acid dehydration, the products did not comigrate with any of the authentic ANF metabolite standards available (see Ref. 14).

ANF metabolism by a reconstituted P-450 system. Incubation mixtures consisted of dilauroylphosphatidylcholine (50 μg/ml), P-450 isozymes (0.005–1 nmol/ml), NADPH-cytochrome *c* reductase (15000 units/nmol of P-450), NADPH (0.5 mM), and [^{3}H]ANF (40 μM; specific activity, 1.65 Ci/mmol) in 100 mM potassium phosphate buffer, pH 7.5, containing 0.5% BSA. After a 5-min preincubation of P-450 isozyme, dilauroylphosphatidylcholine, and NADPH-cytochrome *c* reductase at room temperature, buffer and ANF were added. When used, EH was added to the reconstituted system at a concentration of 20 μg/0.1 nmol of P-450 (i.e., 0.4 nmol/0.1 nmol of P-450). The samples were prewarmed to 37° for 5 min and the reactions were initiated by addition

TABLE 1

Inhibition of microsomal ANF metabolism by P-450 antibodies

Uninduced (0.1 mg/ml) or TCDD-induced microsomes (0.02 mg/ml) were preincubated with specific IgGs at room temperature. Appropriate preimmune IgGs were added as necessary to maintain the same total IgG concentration in each sample. After 5 min, 10 μM [^{3}H]ANF and an NADPH-generating system was added and the samples were incubated for 20 min at 37°. Incubations were terminated and analyzed as described in Materials and Methods.

Microsomes	Antibody				ANF metabolism[a]
			mg of IgG/mg of microsomal protein	*mg of IgG/nmol of P-450c or d*	*% of control*
Uninduced	Anti-P-450c	Lot 1	10	5000	123
			50	25000	108
			100	50000	115
		Lot 2	10	5000	93
			50	25000	100
			100	50000	113
	Anti-P-450d		10	333	120
			50	1666	93
			100	3333	100
TCDD-induced	Anti-P-450c	Lot 1	10	10	54
			50	50	47
			100	100	49
		Lot 2	10	10	33
			50	50	24
			100	100	21
	Anti-P-450d		10	10	94
			50	50	105
			100	100	98

[a] As control we used samples containing only the appropriate preimmune IgGs.

of NADPH. After 15 min of incubation, 5 volumes of water-saturated ethyl acetate were added, and nonpolar ANF metabolites were analyzed.

Analysis of ANF metabolites. Nonpolar metabolites and unreacted ANF were extracted from the incubation mixtures with water-saturated ethyl acetate and analyzed on a reverse phase HPLC column (Dupont Zorbax ODS, 6.2 × 250 mm), using a Varian model Vista 5500 HPLC system. A 30-min linear gradient of 50% to 100% methanol in water, at a flow rate of 1.0 ml/min, was used. Effluent was continuously monitored at 254 nm and the radioactivity was monitored using a Flo-one radioactivity flow detector (Radio-Analytic, Tampa, FL). To distinguish between water-soluble and protein-bound metabolites, the salt concentration in the remaining water phase was increased to 100 mM NaCl and 2 volumes of acetone were added to precipitate the protein. The supernatant and protein pellet were separated and counted in a Minaxi β Tri-Carb (4000 series) liquid scintillation counter. Hydroxy-ANF metabolites were identified by cochromatography with authentic hydroxy-ANF standards, and the relative retention times agreed with previously published data (5). The other peaks were collected and treated for 2 hr at 100° with 4 N H_2SO_4 (5). This acid dehydration converted dihydrodiol and oxide metabolites to hydroxy metabolites. Identification was then based on relative retention times of original peaks and of hydroxy-ANF metabolites formed after acid treatment, using the HPLC chromatographic system described above.

Ethoxyresorufin *O*-deethylation assay. The ethoxyresorufin assay was performed as described by Pohl and Fouts (20). Incubation mixtures contained ethoxyresorufin (1.5 μM; Pierce Chemical Co., Rockford, IL), microsomal protein or a reconstituted P-450 system (P-450 isozyme, NADPH-cytochrome *c* reductase, and dilaurylphosphatidylcholine), immunoglobulin, and NADPH (1 mM) in 100 mM potassium phosphate, 5 mM $MgCl_2$, 0.2% BSA buffer, pH 7.5. After 15 min at 37°, the reaction was terminated by addition of 1 volume of methanol. Samples were centrifuged for 10 min (500 × *g*) and the fluorescence of the supernatants was measured at 585 nm in a Perkin-Elmer LS-5 spectrofluorometer, using an excitation wavelength of 550 nm.

Results

Inhibition of ANF metabolism in rat liver microsomes by anti-P-450c and anti-P-450d. To determine the involvement of P-450c in ANF metabolism, uninduced and TCDD-induced rat liver microsomes were incubated with monospecific anti-P-450c and anti-P-450d before ANF metabolism was assayed. We tested two lots of anti-P-450c and one lot of anti-P-450d. Representative HPLC chromatograms of ANF metabolites are shown in Fig. 1.

Two lots of anti-P-450c inhibited ANF metabolism to different extents in TCDD-induced rat liver microsomes (Table 1, Fig. 2A). The data presented in Fig. 2A represent the results from one experiment in which all antibody preparations were tested at the same time. Each point on the graph represents the average of duplicate incubations. Lot 1 inhibited ANF metabolism by only 50%, whereas lot 2 inhibited metabolism by 80%. In contrast, both lots almost completely inhibited ethoxyresorufin *O*-deethylase activity in a reconstituted system using purified P-450, and both produced similar degrees of inhibition of ethoxyresorufin *O*-deethylase activity in TCDD-induced rat liver microsomes (60–65%) (Fig. 2B). Neither lot of anti-P-450c inhibited ANF metabolism in uninduced rat liver microsomes (Table 1). In contrast to anti-P-450c, anti-P-450d had no effect on ANF metabolism in uninduced or TCDD-induced microsomes (Table 1, Fig. 2). However, this lot of anti-P-450d inhibits the catalytic activity of P-450d, because it inhibits the 17β-estradiol 2-hydroxylase activity of P-450d in a reconstituted system by greater than 98% at 40 mg/nmol of P-450 and inhibits 17β-estradiol 2-hydroxylase activity of TCDD-induced liver microsomes to the level of uninduced liver microsomes (21).

Metabolism of [^{3}H]ANF by a reconstituted P-450 system. When the capacities of purified P-450c, P-450d, and P-450b to metabolize ANF were compared in a reconstituted system, P-450c metabolized ANF to a much greater extent than the other two isozymes (Table 2). The conversion rate of ANF by P-450c was 47 times greater than that by P-450d and 510 times greater than that by P-450b. ANF metabolism by TCDD-

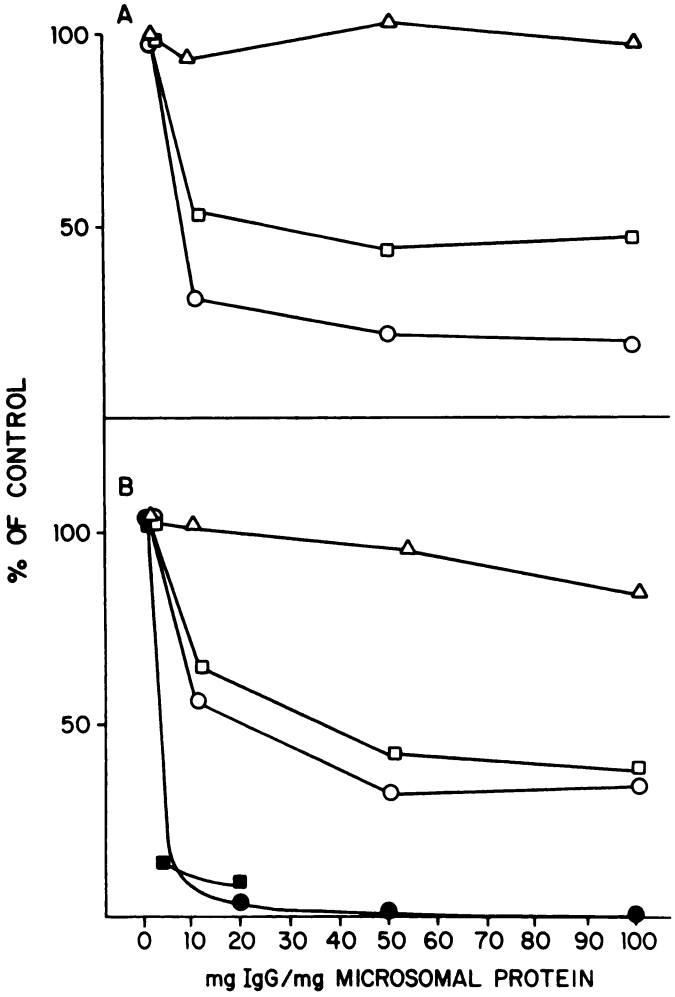

Fig. 2. Inhibitory potency of anti-P-450c and anti-P-450d toward metabolism of ANF (A) or ethoxyresorufin (B). A, Inhibition of ANF metabolism in TCDD-induced microsomes by lot 1 anti-P-450c (□), lot 2 anti-P-450c (○), and anti-P-450d (△). B, Inhibition of ethoxyresorufin metabolism in TCDD-induced microsomes by lot 1 anti-P-450c (□), lot 2 anti-P-450c (○), and anti-P-450d (△). Inhibitory potencies of lot 1 anti-P-450c (■) and lot 2 anti-P-450c (●) toward ethoxyresorufin metabolism were also tested in a P-450c-reconstituted system.

or 3-MC-induced microsomes was also 10 times greater than by uninduced microsomes (Table 2).

With the HPLC method used, the 5,6-oxide-ANF and the 7-hydroxy-ANF coelute. Therefore, to quantify the relative amounts of oxidation at the 7,8- and 5,6-positions, peak fractions were collected and treated with acid (Table 2), as has previously been described. Uninduced microsomes produced approximately equal amounts of the 5,6- and 7,8-oxides. Oxidation at positions other than the 5,6- or 7,8-position were detected only with TCDD- or 3-MC-induced microsomes and with purified P-450c. However, TCDD- and 3-MC-induced microsomes metabolized ANF preferentially at the 7,8-position. When metabolism of ANF by purified P-450s was evaluated in the presence of EH, dihydrodiol formation increased substantially at both the 5,6- and 7,8-positions (Fig. 3B, Table 2). The detection of nonpolar metabolites resulting from oxidation at the 7,8-position (7,8-dihydrodiol, 7-hydroxy-ANF) showed these were a higher percentage (52%) of the total nonpolar metabolites in the presence of EH than in the absence of EH (23%, 90% of which represented 7-hydroxy-ANF) (Table 2).

TABLE 2

ANF metabolism by purified P-450s in a reconstituted system and by rat liver microsomes

	Rate of ANF metabolism	Nonpolar metabolites		
		7,8-position	5,6-position	Other
	nmol/min/nmol of P-450		*% of total*	
Purified cytochromes				
P-450c	11.2 ± 0.8	23[a]	67[b]	10
P-450c + EH	11.2 ± 0.1	52[c]	42[d]	6
P-450d	0.24 ± 0.01	[f]	[f]	ND[g]
P-450b	0.022 ± 0.004	[f]	[f]	ND
	nmol/min/mg of microsomal protein			
Microsomes				
Uninduced	0.5 ± 0.2	50[c]	50[e]	ND
TCDD-induced	4.9 ± 0.6	57[c]	34[d]	9
3-MC-induced	5.5 ± 1.9	55[c]	36[d]	6

[a],[c] Oxidation at 7,8-position resulted in (a) 7-hydroxy-ANF (91%) and 7,8-dihydrodiol (9%) or (c) 7,8-dihydrodiol (100%).

[b],[d],[e] Oxidation at 5,6-position resulted in (b) 5,6-oxide-ANF (96%) and 6-hydroxy-ANF (4%), (d) 5,6-oxide-ANF (84%), 5,6-dihydrodiol (8%), and 6-hydroxy-ANF (8%), or (e) 5,6-oxide-ANF (57%), 5,6-dihydrodiol (18%), and 6-hydroxy-ANF (25%).

[f] Acid treatment to determine amount of 7-hydroxy-ANF and 5,6-oxide-ANF could not be done due to low amount of metabolites.

[g] ND, not detected; limit of detection is 0.1% of total radioactivity.

When total metabolism in the reconstituted system was evaluated in the presence and absence of EH (i.e., including water-soluble and protein-bound metabolites), we found no difference in the amount of water-soluble (polar) metabolites formed (1.4% of total radioactivity for both), but EH decreased the protein-bound radioactivity (9.7 versus 2.8%) (Table 3). These data indicate that EH diminishes electrophilic attack by deactivating 7,8-oxide-ANF.

Previous studies in our laboratory have demonstrated that ANF is a potent clastogen in Chinese hamster ovary cells only when metabolized by TCDD-induced rat liver microsomes (11, 14). Identification of the active ANF metabolite is uncertain; however, there is strong evidence that peak I (Fig. 3B), which has tentatively been identified as the 9,10-dihydrodiol, is responsible for the clastogenic properties of ANF (14). Moreover, the present studies show that the formation of peak I is dependent on the presence of P-450c and EH in the reconstituted system, which is consistent with its identification as a dihydrodiol (Fig. 3).

Discussion

TCDD and 3-MC increase ANF metabolism, and only three isozymes of P-450 (c, d, and a) are known to be induced by TCDD (17, 21). ANF metabolism is increased approximately 10-fold by TCDD. This increase is less than the increase in ethoxyresorufin *O*-deethylase activity and less than the >100-fold increase in P-450c (22) seen after TCDD treatment. However, this increase is consistent with the 13-fold increase in aryl hydrocarbon hydroxylase activity, another P-450c-mediated enzyme (22). Domin and Philpot (23) have attributed a similar discrepancy in the TCDD-induced increase in aryl hydrocarbon hydroxylase activity and isozyme 6 in rabbit lung to limiting amounts of cytochrome *c* reductase, which appear to affect catalytic activity toward BP more than activity toward ethoxyresorufin. Therefore, the magnitude of the increase in ANF metabolism is consistent with the increase in another P-

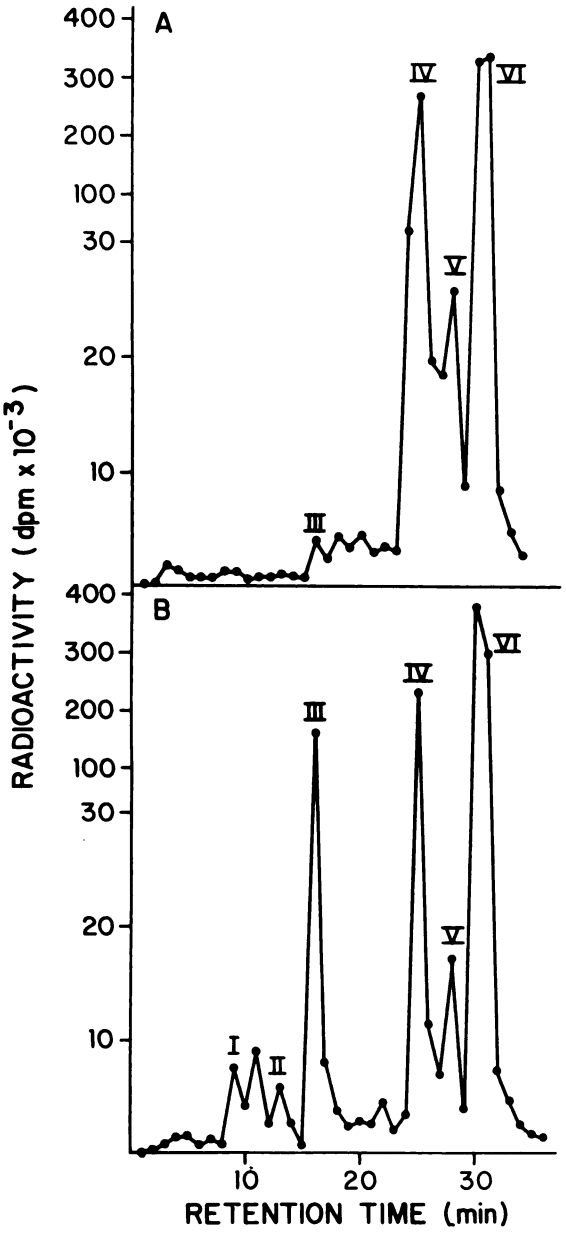

Fig. 3. Metabolism of ANF in a reconstituted system with P-450c, in the absence or presence of epoxide hydrolase. HPLC chromatographs of ANF metabolites formed following 15-min incubation with a P-450c-reconstituted system in the absence of epoxide hydrolase (A) and in the presence of epoxide hydrolase (20 μg/0.1 nmol of P-450c) (B).

TABLE 3

Distribution of ANF metabolites in nonpolar, polar, and protein-bound fractions in a reconstituted system

Values represent the mean ± standard deviation of three experiments. The conditions for the incubations and separation of fractions are described in Materials and Methods.

Reconstituted system	Metabolites			
	Nonpolar	Polar	Protein-bound	Unreacted ANF
	% of total radioactivity			
P-450c – EH	37.4 ± 2.3	1.4 ± 0.3	9.7 ± 0.8	51.6 ± 2.8
P-450c + EH	40.2 ± 0.4	1.5 ± 0.3	2.8 ± 0.7	55.5 ± 0.6

450c-mediated enzymatic activity. Vyas *et al.* (6) reported only 16% inhibition of ANF metabolism, using 3-MC-induced microsomes, by anti-P-450c and no inhibition by anti-P-450d or anti-P-450a.

Our data indicate that P-450c is the primary cytochrome responsible for the increase in ANF metabolism. However, the amount of inhibition by anti-P-450c depended on the batch of antibody used. We used two different lots of anti-P-450c, which appeared to be equally effective in inhibiting ethoxyresorufin *O*-deethylase, another P-450c-dependent enzyme. However, one lot of anti-P-450c inhibited ANF metabolism by only 50%, whereas the other lot produced 80% inhibition. ANF metabolism by uninduced rat liver microsomes was not inhibited by anti-P-450c or anti-P-450d, indicating that other constitutive isozymes are responsible for this activity in control microsomes. This is not surprising, because P-450c accounts for less than 0.5% of the total P-450 in control liver microsomes (17).

It seems likely that different batches of anti-P-450c contain different proportions of antibodies to particular substrate-binding epitopes of P-450c. In our studies, monospecific polyclonal antibodies to P-450c and P-450d have been absorbed to remove cross-reactivity. Because P-450c and P-450d are 70% similar in their deduced amino acid sequence, antibodies to many epitopes will be removed. The epitopes involved in substrate binding or metabolism may differ for different substrates. Similarly, monoclonal antibodies might affect binding of one substrate to P-450c but not of another substrate. Alternatively, antibodies to P-450c may produce greater inhibition of substrate P-450c interaction when their binding affinity is greater than the affinity of the cytochrome for the substrate. Nesnow (7) reported that 10 μM ANF can inhibit 50% of the metabolism of 60 μM BP by 3-MC-induced microsomes, which shows that the affinity of P-450c for ANF is greater than that between P-450c and BP. Therefore, it would seem possible that, if different preparations of anti-P-450c possess different affinities for P-450c, they could inhibit metabolism of one substrate in a similar way (e.g., ethoxyresorufin) and still give different degrees of inhibition of ANF metabolism. However, from our results we cannot rule out the possibility that the lot 2 antibody was just most inhibitory against both ethoxyresorufin and ANF.

Analysis of ANF metabolism in a reconstituted system using purified P-450s showed that P-450c was 47 and 510 times more active than P-450d and P-450b, respectively, supporting our conclusion that P-450c is the primary cytochrome involved in ANF metabolism in TCDD-induced liver microsomes. However, there were apparent differences between the metabolism of ANF by TCDD-induced microsomes and its metabolism in a reconstituted system containing P-450c in the absence of EH, which was corrected to some extent by the addition of EH. In the reconstituted system containing P-450c, we observed a 3.5-fold increase in the radioactivity binding to protein in the absence of EH. This increase in protein binding accounted for the observed difference in oxidization at the 7,8-position in the reconstituted system in the absence of EH, compared with metabolism in the presence of EH. The 7,8-oxide-ANF is unstable but an excellent substrate for EH. In the absence of EH, the 7,8-oxide-ANF can either nonenzymatically rearrange to the 7-hydroxy-ANF or react with nucleophilic macromolecules such as protein. In the presence of EH, apparent increases in nonpolar metabolites of ANF at the 7,8-position are observed, because almost all the 7,8-oxide-ANF is further metab-

olized to the stable 7,8-dihydrodiol and this reaction prevents binding to protein. In contrast, 5,6-oxide-ANF is a stable metabolite and a poor substrate for EH (6); therefore, its level appears to be unaffected by EH.

The formation of peak I by P-450c in a reconstituted system is dependent on the presence of EH. The importance of this ANF metabolite for the clastogenic properties of ANF has been previously discussed (14). The identity of this peak is not known; however, it has been tentatively identified by acid dehydration as 9,10-dihydrodiol. Methods such as mass spectrometry or NMR spectrometry will be necessary for conclusive identification of the peak I ANF metabolite.

In conclusion, our data suggest that P-450c is the cytochrome responsible for ANF metabolism in TCDD-induced rat liver microsomes. P-450c also appears to be responsible for the increased formation of peak I, believed to be the clastogenic metabolite of ANF.

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