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ENHANCED ARYL HYDROCARBON HYDROXYLASE ACTIVITY AFTER INTERACTION BETWEEN SOLUBILIZED CYTOCHROME P-448 AND MICROSOMES LOW IN ENDOGENOUS CYTOCHROME P-450

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Abstract—The effects of cumene hydroperoxide on microsomal mixed-function oxidase components and enzyme activities were determined. *In vitro* cumene hydroperoxide treatment decreased cytochrome P-450 content, benzphetamine *N*-demethylase activity and aryl hydrocarbon hydroxylase activity of hepatic and renal microsomes from adult male and female rats, and of hepatic microsomes from fetal rats. Cumene hydroperoxide-treated microsomes, as well as fetal liver and adult renal microsomes, which are naturally low in cytochrome P-450 and mixed-function oxidase activity, were used to incorporate partially purified hepatic cytochrome P-448 isolated from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-pretreated immature male rats. This resulted in an enhanced rate of benzo[*a*]pyrene hydroxylation. Aryl hydrocarbon hydroxylase activity was increased 12-, 26-, 31- and 53-fold when 1.0 nmole of partially purified cytochrome P-448 was incubated with fetal liver microsomes, microsomes from kidney cortex of female rats, and cumene hydroperoxide-pretreated hepatic microsomes from female and male rats, respectively. The increased rate of benzo[*a*]pyrene hydroxylation was linear with cytochrome P-448 over the range 0.25 to 1.0 nmole. Because cumene hydroperoxide-pretreated microsomes from male rat liver and the hepatic and renal microsomes from female rats have a combination of high NADPH-cytochrome *c* reductase activity and low mixed-function oxidase activity, they are an attractive choice for catalytic studies of the interaction between cytochrome P-448 and microsomes.

Partially purified microsomal electron transport proteins, such as cytochrome P-448† [1, 2], cytochrome P-450 [1, 3], cytochrome b_5 [4–7], NADPH-cytochrome *c* reductase (NADPH-cytochrome P-450 reductase) [3, 8] and NADH-cytochrome b_5 reductase [7, 9], have been incorporated into microsomal membranes and shown to be capable of their normal electron transport functions. Interpreting the functions of microsomes which have incorporated such exogenous proteins is sometimes difficult, because the incorporated exogenous and the endogenous proteins function similarly. Since at least four [10, 11] and probably six [12, 13] different forms of cytochrome P-450 have been identified, the problem of endogenous cytochrome P-450-mediated enzyme activity is particularly complex. The enzymatic specificity of the microsomal mixed-function oxidase (MFO)‡ system resides in the hemoprotein [14, 15] portion of this three component [16–18] (cytochrome P-450, NADPH-cytochrome P-450 reductase and phospholipid) membrane bound enzyme system.

Since endogenous cytochrome P-450 and incorporated exogenous cytochrome P-450 are both enzymatically active, it is desirable to incorporate exogenous cytochrome P-450 into microsomes with endogenous cytochrome P-450-mediated enzyme activities as low as possible. This allows for the smallest possible 'blanks' and the greatest sensitivity for enzymatically detecting incorporated exogenous cytochrome P-450. Because of the high endogenous activity of the various forms of cytochrome P-450, linoleic acid hydroperoxide (LAHP) pretreatment has been used to decrease endogenous cytochrome P-450 and MFO enzyme activity [1, 19] prior to incorporation of exogenous cytochrome P-450 and P-448 [1]. However, LAHP is not commercially available and must be synthesized and purified [20] by investigators. Cumene hydroperoxide, a commercially available hydroperoxide, also lowers microsomal cytochrome P-450 content [21]. Glutathione partially prevents CHP- [21] and LAHP- [19, 22] induced decreases in cytochrome P-450. Organic hydroperoxides and cytochrome P-450 have an interesting reciprocal relationship as many heme compounds [20] degrade LAHP while both LAHP and CHP decrease cytochrome P-450 levels. CHP has also been used as a substitute for NADPH and molecular oxygen for short incubations (1–2 min) in some reconstituted MFO systems [23–27]. NADPH, in the presence of iron and ADP, causes lipid peroxidation and degradation of hepatic microsomal

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† In this paper, cytochromes P-450 and P-448 are used to denote the hepatic microsomal CO-binding pigments found in control and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-pretreated rats, respectively. When reduced and complexed with carbon monoxide, both cytochrome P-450 and P-448 exhibit a Soret maximum in the 450 nm region and cannot be easily spectrally separated. Cytochrome P-448 is also called cytochrome P_1-450.

‡ Abbreviations used are MFO,mixed-function oxidase; LAHP, linoleic acid hydroperoxide; CHP, cumene hydroperoxide; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and AHH, aryl hydrocarbon hydroxylase.

cytochrome P-450 *in vitro* [28, 29]. Both CHP and LAHP cause lipid peroxidation in liver microsomes and it has been stated that LAHP is more potent than CHP in lipid peroxidation [30]. Other evidence, however, suggests that lipid peroxidation cannot account for 50 per cent of the destruction of cytochrome P-450 observed with hydroperoxides [31] and that LAHP can cause decreases in hepatic microsomal P-450 without any lipid peroxidation [19].

In the present studies the effects of CHP treatment on microsomal cytochrome P-450, cytochrome b_5, NADPH-cytochrome *c* reductase, benzphetamine *N*-demethylase and AHH have been determined. The CHP-treated, cytochrome P-450-denatured microsomes, as well as rat microsomes naturally low in cytochrome P-450 and MFO activity because of organ [32], sex [33] and ontogenic [34] differences, were used to incorporate exogenous cytochrome P-448 and to determine enhanced* rates of benzo[*a*]pyrene hydroxylation. Hepatic and renal microsomal membranes high in NADPH-cytochrome *c* reductase activity and low in cytochrome P-450 and MFO enzyme activity were sought so that high ratios of enhanced AHH activity to endogenous AHH activity could be obtained. The suitability of these microsomal membranes for cytochrome P-448 interaction studies was evaluated by comparing their endogenous NADPH-cytochrome *c* reductase activities, endogenous AHH activities and their enhanced AHH rates after incubation with exogenous cytochrome P-448. In addition, CHP pretreatment and NADPH-dependent lipid peroxidation have been compared as methods of lowering endogenous cytochrome P-450 content, decreasing MFO activity and increasing the rate of benzo[*a*]pyrene hydroxylation following incorporation of exogenous cytochrome P-448. Cumene hydroperoxide-induced hemoprotein degradation, NADPH-dependent lipid peroxidation and use of microsomal membranes normally low in cytochrome P-450 and MFO activities are discussed with respect to the ratio of MFO components, lipid peroxidation and enhanced AHH activity following incubation with exogenous cytochrome P-448.

MATERIALS AND METHODS

Chemicals. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (99 per cent pure) was synthesized by Dr. Kun Chae in the Environmental Chemistry and Biology Branch of NIEHS. Benzo[*a*]pyrene, isocitric acid, isocitric dehydrogenase (type I), NADPH, $NADP^+$, NADH, ATP, antimycin A, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (type XV), semicarbazide, dithiothreitol and sodium cholate were obtained from the Sigma Chemical Co. (St. Louis, MO). Cumene hydroperoxide and glutathione (reduced) were purchased from Matheson Coleman & Bell (Norwood, OH) and CalBiochem (San Diego, CA), respectively. Benzphetamine hydrochloride and [^{14}C]benzphetamine were obtained from UpJohn Research Laboratories (Kalamazoo, MI) and New England Nuclear (Boston, MA) respectively. Diethylaminoethylcellulose (DE 52) was purchased from Scientific Products (McGraw Park, IL). Bio-Rad (Richmond, CA) supplied the calcium phosphate gel. Emulgen 911 came from the Kao-Atlas Co. (Tokyo, Japan), while sephadex LH-20 was purchased from Pharmacia (Piscataway, NJ). The 3-hydroxybenzo[*a*]pyrene was a gift of Dr. Hans Falk (NIEHS).

Animals and preparation of microsomes. Male and female Sprague–Dawley rats (CD strain) (200–275 g), obtained from Charles River Laboratories (Wilmington, MA) were housed four per glass cage with food and water *ad lib.* Fetal livers were obtained from 21-day pregnant rats. After the animals were decapitated the livers and kidneys were excised, weighed and homogenized with seven strokes of Potter–Elvehjem homogenizer fitted with a Teflon pestle. Livers were homogenized in 5 vol of 1.15% KCl containing 0.02 M Tris–HCl, pH 7.5. Liver homogenates were centrifuged at 9000 *g* for 15 min, and the microsomal fraction was prepared from the 9000 *g* supernatant solution by centrifugation at 165,000 *g* for 35 min. Kidney microsomes were prepared by the method of Jakobsson [35]. The microsomal pellets were resuspended in 0.05 M Tris–HCl buffer with 0.15 M KCl, pH 7.5 (livers), or 0.25 M sucrose (kidneys) and either kept in ice or incubated with 2.0 mM CHP for 20 min at 37°. The microsomal suspensions (3.7–5.0 mg protein/ml) were then made 2.0 mM† with glutathione dissolved in Tris–KCl buffer and incubated at 37° for an additional 3 min. CHP and glutathione solutions (both 20.0 mM in 0.05 M Tris–0.15 M KCl, pH 7.5) were prepared fresh daily just prior to use. The microsomes were cooled in an ice water bath for 10 min and recentrifuged at 165,000 *g* for 35 min. In some experiments, liver microsomes (1.0–1.3 mg protein/ml) were also incubated with NADPH (1.25 mM), ATP (0.85 mM) and Fe(II)SO_4 (10 μM) at 37° for 20 min [36], and subsequently cooled and recentrifuged as described for cumene hydroperoxide-treated microsomes. The final microsomal pellets were gently resuspended in 0.05 M Tris–HCl buffer, pH 7.5, using a Potter-Elvehjem homogenizer with a Teflon pestle so that each 1 ml of final suspension was obtained from approximately 1 g of starting tissue.

Partial purification of rat hepatic cytochrome P-448. Male rats (55–75 g) were given 10 μg/kg of TCDD *per os* 1 week before being killed. Cytochrome P-448 was solubilized from freshly prepared microsomes with 1% sodium cholate by the method of Lu and Levin [37]. The cholate to protein ratio of the solubilized P-448 was adjusted to 1.3, and the precipitate that formed between 35 and 45% saturation of ammonium sulfate [38] was collected and dissolved in 0.005 M potassium phosphate buffer, pH 7.7, containing 20% glycerol, 0.1 mM dithiothreitol, 0.1 mM EDTA and 0.1% cholate. After ultracentrifugation at 160,000 *g* for 1 hr the solubil-

* Enhanced AHH activity refers to the increase in AHH activity observed when partially purified cytochrome P-448 is incubated with microsomes. Previous studies of Yang and Strickhart [2], Yang [1] and Miwa *et al.* [3] have shown that exogenous cytochrome P-450 and P-448 can become incorporated into the microsomal membrane and can receive electrons from NADPH-cytochrome *c* reductase [1] and catalyze MFO reactions [1–3].

† To ensure the destruction of CHP, higher concentrations of glutathione may be used [1, 19, 21]. CHP is also degraded by the endogenous microsomal cytochrome P-450 and slowly inactivated by the Tris–HCl buffer itself.

ized cytochrome P-448 was dialyzed against this buffer overnight. The dialysate was treated with 1 mg Emulgen 911/mg protein and applied to a DEAE-cellulose column as described by Levin *et al.* [39]. Cytochrome P-448 was eluted with 0.05 M potassium phosphate buffer containing 20% glycerol and 0.1% Emulgen 911 and quickly passed through a Sephadex LH-20 column to reduce the Emulgen 911 content of the cytochrome P-448 preparation [39]. The partially purified cytochrome was extracted from 20 mM potassium phosphate buffer with calcium phosphate gel, washed twice with 100 mM potassium phosphate buffer and eluted from the gel with 250 mM potassium phosphate buffer [37]. All three potassium phosphate buffers (pH 7.7) contained 20% glycerol, 0.1 mM EDTA and dithiothreitol, and 0.1% cholate.

The sample was dialyzed overnight against 0.005 M potassium phosphate containing 20% glycerol, 0.1 mM EDTA and dithiothreitol, and 0.1% cholate, and redialyzed again against the same buffer containing 0.05% cholate [37]. The dialysate was centrifuged at 20,000 *g* for 15 min, assayed for cytochromes P-450, P-420 and b_5, NADPH-cytochrome *c* reductase and protein, and then stored frozen under N_2 at −80°. The final cytochrome P-488 preparation, purified 2.4-fold from the starting microsomes (recovery 4.3 per cent), contained 2.72 nmoles cytochrome P-448/mg protein. This preparation had little or no cytochrome b_5, cytochrome P-420 or NADPH-cytochrome *c* reductase. Although the partially purified cytochrome P-448 was not enzymatically active *per se*, it was capable of catalyzing benzo[*a*]pyrene hydroxylation when incubated with microsomes, or when reconstituted [40] with NADPH-cytochrome *c* reductase and dilauroyl phosphatidylcholine. (The turnover number was 0.8 nmole 3-hydroxybenzo[*a*]pyrene/min/nmole cytochrome 448.)

Enhanced aryl hydrocarbon hydroxylase activity and enzyme assays. Solubilized cytochrome P-448 (1.0–4.0 nmoles) and various microsomes (0.25–0.60 mg protein) were incubated at 37° for 30 min [1] in 250 μl of 0.05 M Tris–HCl buffer, pH 7.5. Then 0.05 M Tris–HCl buffer, pH 7.5, containing 5 mM Mg^{2+} and 5 μM Mn^{2+}, as well as $NADP^+$ (1 mM), isocitrate dehydrogenase (0.36 units) and isocitrate (6 mM) was added to a final volume of 1.0 ml, and aryl hydrocarbon hydroxylase activity was measured by the fluorometric assay as described by DePierre *et al.* [41]. Fluorescence of the metabolites of benzo[*a*]pyrene formed during incubation with microsomes is expressed relative to a 3-hydroxybenzo[*a*]pyrene internal standard. The radiometric AHH assay of DePierre *et al.* [41], as modified by Nesnow *et al.* [42] to include the arene oxide metabolites of benzo[*a*]pyrene, was also used. NADPH-cytochrome *c* reductase was assayed by the method of LaDu *et al.* [43] modified by the inclusion of 1.6 μM antimycin A in the incubation media. One unit of reductase activity is 1 nmole of cytochrome *c* reduced per min. [^{14}C]Formaldehyde formed from the *N*-demethylation of [^{14}C]benzphetamine was assayed by the procedure of Thomas *et al.* [44]. Cytochrome b_5 was reduced by NADH and spectrally quantified using an extinction coefficient of 185 mM^{-1} cm^{-1} for the absorption difference between 424 and 410 nm [45]. Dithionite was used to reduce cytochrome b_5 in the partial purification of cytochrome P-448 procedure. The total cytochrome P-450 content of microsomes was determined by the carbon monoxide difference spectra of dithionite-reduced microsomal suspensions. An extinction coefficient of 91 mM^{-1} cm^{-1} was used in P-450 calculations [45]. Cytochrome P-420 content was determined by the method of Omura and Sato [46]. Protein determinations were performed by the method of Lowry *et al.* [47], and statistical levels of significance were determined by using the two-sided Student's *t*-test [48].

RESULTS

Effects of cumene hydroperoxide on microsomal hemoprotein content and enzyme activities. Cumene hydroperoxide treatment decreased the cytochrome P-450 content of all rat tissues studied while not affecting cytochrome b_5 levels. Results of these studies are shown in Table 1. *In vitro* cumene hydroperoxide treatment lowered hepatic cytochrome P-450 by 80 and 75 per cent in hepatic microsomes from male and female rats, respectively. Similarly, the cytochrome P-450 content of 21-day fetal liver microsomes was decreased 83 per cent. However, renal microsomal cytochrome P-450 content was lowered only 50 per cent by CHP. CHP treatment also decreased the cytochrome P-420 content of hepatic microsomes previously treated with chloroform (data not shown). Hepatic microsomal NADPH-cytochrome *c* reductase activity was unaffected by cumene hydroperoxide pretreatment. However, CHP treatment decreased renal microsomal NADPH-cytochrome *c* reductase activity by 51 and 46 per cent in male and female rats, respectively.

The data of Table 1 also show that cytochrome P-450-mediated enzyme activities were greatly decreased by prior treatment of microsomes with cumene hydroperoxide. Benzphetamine *N*-demethylase activities were decreased 85–90 per cent by cumene hydroperoxide, when compared to the values obtained from microsomes kept on ice. Cumene hydroperoxide treatment lowered aryl hydrocarbon hydroxylase activities by 90–98 per cent in renal and hepatic microsomes of adult rats. Twenty-one-day fetal liver microsomal AHH activity was decreased by about one-half after treatment with 2.0 mM cumene hydroperoxide. Although incubation at 37° for 30 min *per se* had only minor effects on cytochrome P-450 content and benzphetamine *N*-demethylase activity, the AHH activities were increased about 130 per cent incubation (data not shown).

Enhanced aryl hydrocarbon hydroxylase activity following incubation of partially purified cytochrome P-488 with various microsomal membranes. Microsomes naturally low in AHH activity and microsomes pretreated with CHP (and thus partially depleted of cytochrome P-450) were used to demonstrate enhanced rates of catalysis after *in vitro* incubation with exogenous cytochrome P-448. Results of these studies are shown in Table 2. The AHH activities of all nine microsomal samples that were low in endogenous AHH activity were increased from 4-

Table 1. Effects of cumene hydroperoxide on rat microsomal hemoprotein content and mixed function oxidase enzyme activities*

Microsomal sample	Cumene hydroperoxide	NADPH-cytochrome *c* reductase (nmoles/mg/min)	Cytochrome b_5 (nmoles/mg)	Cytochrome P-450 (nmoles/mg)	Benzphetamine *N*-demethylase (nmoles formaldehyde/mg/min)	Aryl hydrocarbon hydroxylase (pmoles/mg/min)
Adult male liver	−	120 ± 9.3	0.32 ± 0.02	1.06 ± 0.11	4.77 ± 0.38	505 ± 700
Adult male liver	+	126 ± 5.6	0.40 ± 0.03	0.21 ± 0.06†	0.46 ± 0.06‡	12 ± 4.7‡
Adult female liver	−	107 ± 5.5	0.36 ± 0.04	0.87 ± 0.08	1.21 ± 0.10	57 ± 14
Adult female liver	+	99 ± 7.2	0.44 ± 0.04	0.22 ± 0.03‡	0.09 ± 0.06‡	5.2 ± 0.4†
Fetal liver	−	17 ± 2.1	0.10 ± 0.02	0.06 ± 0.003	0.13 ± 0.03	3.2 ± 0.7
Fetal liver	+	16 ± 0.7	0.07 ± 0.01	0.01 ± 0.01§	0.02 ± 0.01†	1.7 ± 0.8
Adult male kidney	−	35 ± 0.9	0.03 ± 0.03	0.17 ± 0.02	0.09 ± 0.03	14 ± 2.6
Adult male kidney	+	17 ± 4.5†	0.06 ± 0.02	0.09 ± 0.01§	0.006 ± 0.003§	1.4 ± 0.3†
Adult female kidney	−	37 ± 4.1	0.02 ± 0.02	0.17 ± 0.01	0.08 ± 0.02	17 ± 4.5
Adult female kidney	+	20 ± 1.9†	0.03 ± 0.01	0.08 ± 0.01‡	ND‖	0.6 ± 0.1†

* Rat microsomes were prepared and either left on ice or incubated with cumene hydroperoxide (2.0 mM) at 37° for 20 min followed by glutathione (2.0 mM) for 3 min. Microsomal suspensions were cooled, repelleted and suspended in 0.05 M Tris–HCl, pH 7.5, and assayed for specific hemoprotein content and mixed function oxidase enzyme activity described in Materials and Methods. Values are the means ± S.E. of three or more determinations with the levels of statistical significance (vs no cumene hydroperoxide) indicated for each microsomal sample.

† $P < 0.01$.

‡ $P < 0.001$.

§ $P < 0.05$.

‖ Not detectable.

Table 2. Enhanced aryl hydrocarbon hydroxylase activity following incubation of partially purified cytochrome P-448 with various microsomal membranes*

Microsomal sample	Cumene hydroperoxide	Microsomal protein (mg)	Microsomal NADPH-cytochrome c reductase (nmoles/min)	Aryl hydrocarbon hydroxylase (pmoles 3-hydroxybenzo[a] pyrene/min/unit reductase) No addition	Plus 1.0 nmole cytochrome P-448
Adult male liver	−	0.31	30.1	17.4 ± 2.0	18.4 ± 0.77†
Adult male liver	+	0.26	17.9	0.20 ± 0.03	10.6 ± 0.52‡
Adult female liver	−	0.30	27.3	1.67 ± 0.15	7.1 ± 0.35‡
Adult female liver	+	0.23	18.6	0.26 ± 0.04	8.2 ± 0.36‡
Fetal liver	−	0.55	4.3	0.44 ± 0.10	5.2 ± 0.24‡
Fetal liver	+	0.59	4.1	0.30 ± 0.17	2.3 ± 0.20‡
Adult male kidney	−	0.37	12.2	0.16 ± 0.04	6.2 ± 0.16‡
Adult male kidney	+	0.40	9.6	0.17 ± 0.05	1.0 ± 0.05‡
Adult female kidney	−	0.35	11.1	0.39 ± 0.01	10.2 ± 0.50‡
Adult female kidney	+	0.33	7.6	0.09 ± 0.04	1.27 ± 0.03‡

* Control or cumene hydroperoxide-treated microsomes (0.23–0.60 mg protein) were incubated either with or without 1.0 nmole cytochrome P-448 at 37° for 30 min. The aryl hydrocarbon hydroxylase activity was determined as described in Materials and Methods and expressed as pmoles 3-hydroxybenzo[a]pyrene per min per unit NADPH-cytochrome c reductase activity. AHH values are the means ± S.E. of three or more determinations with the statistical level of significance (vs no addition) given.

† Incubation with 4.0 nmoles cytochrome P-448 resulted in an AHH activity of 15.5 ± 1.1.

‡ $P < 0.001$.

to 53-fold after incubation with 0.1 nmole of cytochrome P-448. In contrast, the AHH activity of microsomes from adult male liver, which possessed the highest endogenous AHH activity, was not significantly increased by incubation with either 1.0 or 4.0 nmoles of exogenous cytochrome P-448. When microsomes from CHP-pretreated male rat liver were incubated with 1.0 nmole of exogenous P-488, the AHH activity rose from 0.20 to 10.6 pmoles 3-hydroxybenzo[a]pyrene formed/min/unit reductase activity (a 53-fold increase). A 31-fold increase in AHH activity was observed after cumene hydroperoxide-treated liver microsomes from female rats were allowed to interact with 1.0 nmole of cytochrome P-448 for 30 min at 37°. After microsomes from female rat liver and cytochrome P-448 were incubated together at 37° for 30 min, 99 per cent of the enhanced AHH activity was removed from the suspension by ultracentrifugation at 105,000 *g* for 60 min. Renal microsomes from male and female rats hydroxylated benzo[a]pyrene 39- and 26-fold faster than did microsomes incubated without cytochrome P-448. However, CHP pretreatment reduced both the size of the increase and the per cent increase in AHH activity following cytochrome P-448 incubation with fetal rat liver and adult kidney microsomes. The enhanced AHH rates for CHP-treated microsomes may be divided by the AHH rate for untreated microsomal samples. If CHP treatment had no effect on either exogenous cytochrome P-448 incorporation into microsomes of the coupling of incorporated exogenous cytochrome P-448 with endogenous NADPH-cytochrome *c* reductase, the relative enhanced AHH rate would be 1.0.* Increases or decreases in the relative enhanced AHH rate may mean that incorporation of exogenous cytochrome P-448 or the coupling of incorporated exogenous cytochrome P-448 with endogenous NADPH-cytochrome *c* reductase has been altered by prior treatment of the microsomal samples with CHP. The relative enhanced AHH rates were 1.46, 0.42, 0.13 and 0.12 for female adult liver, fetal liver, male kidney and female kidney, respectively.

Because of the different rate enhancement observed when 1.0 nmole of cytochrome P-448 was incubated with different microsomal samples, it was important to demonstrate that the increase in AHH activity was linear with the amount of exogenous cytochrome used in the incubation. Results of these

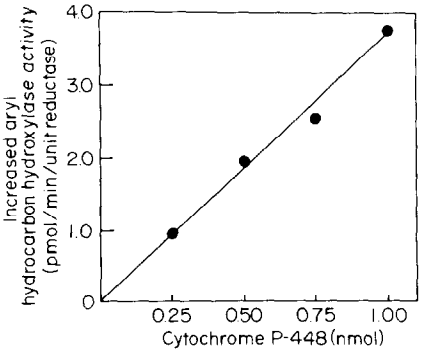

Fig. 1. Linearity of increased microsomal aryl hydrocarbon hydroxylase activity with exogenous cytochrome P-448. Hepatic microsomes from female rats were incubated at 37° for 30 min with 0.25 to 1.00 nmole of cytochrome P-448 in 250 μl of 0.05 M Tris–HCl, pH 7.5. The increased aryl hydrocarbon hydroxylase activity due to incorporation of enzymatically active cytochrome P-448 was then determined.

* The term relative enhanced AHH rate refers to the enhanced AHH rate of CHP-treated microsomes divided by the enhanced AHH rate for untreated microsomes from the same source:

$$\frac{[(\text{AHH rate of CHP-treated microsomes + cytochrome P-488}) - (\text{AHH rate of CHP-treated microsomes})]}{[(\text{AHH rate of microsomes + cytochrome P-448}) - (\text{AHH rate of microsomes})]}$$

studies are shown in Fig. 1. The increase in AHH activity observed when cytochrome P-448 was incubated with hepatic microsomes from female rats is linear with cytochrome P-448, at least in the range 0.25 to 1.0 nmole cytochrome P-448.

The fluorometric AHH assay used in this study determines only the highly fluorescent phenolic metabolites of benzo[*a*]pyrene such as 3-hydroxybenzo[*a*]pyrene. For this reason, experiments were also performed using a radiometric assay of benzo[*a*]pyrene monooxygenase (AHH) which can quantitate all polar metabolites including arene oxide metabolites of benzo[*a*]pyrene [42]. This method is highly useful in situations where epoxide hydrase activity may not be sufficient to convert epoxides into more polar metabolites. After incubation with 1.0 nmole of cytochrome P-448, the AHH activity of female rat liver microsomes was increased from 2.6 to 16.7 pmoles of polar products/min/unit reductase. The 6.3-fold increase in AHH activity using the radiometric assay is greater than the 4.3-fold increase using the fluorometric assay (Table 2). This suggests that incubation with exogenous cytochrome P-488 increases the formation of the non-phenolic metabolites of benzo[*a*]pyrene to a greater extent than it does the fluorescent phenolic metabolites.

It is a matter of convenience for investigators to be able to use frozen rather than freshly prepared microsomal samples. Thus, experiments were performed in which cytochrome P-448 and either fresh or frozen microsomal suspensions from female rat liver were incubated together and the enhanced AHH rates were compared. Results of this study are given in Table 3. Although freezing, storage and thawing had no effect on microsomal protein, the specific activity of NADPH-cytochrome *c* reductase decreased from 121 to 102 and 93 nmoles/mg/min 8 and 16 days later. Freezing decreased the endogenous AHH activity by more than half on both per incubation and per unit reductase basis. The increase in AHH activity following incubation with cytochrome P-488 was higher in frozen than in fresh microsomes. After incubation with cytochrome P-448, the enhanced AHH activity rose from 3.3 to 4.9 and 6.0 pmoles/min/unit reductase in fresh microsomes and in microsomes frozen for 8 and 16 days, respectively.

Comparison of the effects of cumene hydroperoxide pretreatment and NADPH-dependent lipid peroxidation on microsomal hemoprotein content, MFO enzyme activity and increased AHH activity. Because of the possible involvement of lipid peroxidation in CHP-induced decreases in microsomal cytochrome P-450 content and MFO enzyme activity, the effects of CHP treatment and NADPH-dependent lipid peroxidation on microsomal cytochrome P-450, MFO enzyme activity and enhanced AHH activity were compared. Results of this study are shown in Table 4. CHP treatment decreased hepatic microsomal protein by 10–40 per cent, while NADPH treatment reduced Lowry protein concentrations by about 50 per cent. The large decreases in microsomal protein concentrations of NADPH-treated microsomes were confirmed by use of a second protein assay [49]. The percentage of the total protein found in the supernatant fraction of the second microsomal pellet was 26, 37 and 57 per cent for control, CHP- and NADPH-treated samples. CHP treatment did not solubilize either cytochrome P-450 or b_5 from the microsomal membrane. Although cytochrome b_5 was unaffected, 5 per cent of the total microsomal cytochrome P-450 content was solubilized by treatment with NADPH. In NADPH-treatment microsomes, the specific activity of NADPH-cytochrome *c* reductase and cytochrome b_5 content per mg microsomal protein were significantly increased, although the total NADPH-cytochrome *c* reductase activity and cytochrome b_5 content per *g* liver were not increased. *In vitro* treatment of microsomes with NADPH, ATP and ferrous sulfate also significantly decreased, but not to as large an extent as did CHP treatment, the cytochrome P-450 content (41 per cent), benzphetamine *N*-demethylase activity (80 per cent) and AHH activity (97 per cent). Both CHP and NADPH pretreatment greatly aid the demonstration of enhanced AHH activity following the incubation of exogenous cytochrome P-448 with hepatic microsomes from male rats. When incubated with

Table 3. Comparison of the increased AHH activity of fresh and frozen microsomes incubated with 1.0 nmole cytochrome P-448*

Microsomal sample			Aryl hydrocarbon hydroxylase (pmoles/min)			
			Per incubation		Per unit reductase	
Storage	Protein (mg)	NADPH-cytochrome *c* reductase (nmoles/min)	No addition	Plus cytochrome P-488	No addition	Plus cytochrome P-488
Fresh	0.38	46.0	61 ± 1.6 (2)	153 ± 9 (2)	1.32 ± 0.04 (2)	3.28 ± 0.20 (2)
Frozen 8 days	0.39	40.0	25 ± 1.6† (4)	199 ± 11 (4)	0.61 ± 0.04‡ (4)	4.91 ± 0.26§ (4)
Frozen 16 days	0.36	33.5	22 ± 1.7† (4)	205 ± 4.8‡ (4)	0.65 ± 0.05‡ (4)	6.02 ± 0.14† (4)

* Fresh microsomes from a female rat were prepared and incubated either with or without 1.0 nmole of partially purified cytochrome P-448 as described in Materials and Methods. After this microsomal suspension (15.4 mg/ml in 0.05 M Tris–HCl, pH 7.5) was frozen under N_2 and stored at −20°; the experiment was repeated 8 and 16 days later. Values are means ± S.E. with the number of determinations indicated. Statistical levels of significance are for comparison with fresh microsomes.

† $P < 0.001$.

‡ $P < 0.01$.

§ $P < 0.05$.

Table 4. Comparison of cumene hydroperoxide pretreatment and NADPH-dependent lipid peroxidation on male rat hepatic microsomal hemoprotein content, mixed function oxidase activity and the ability to incorporate catalytically active cytochrome P-448*

Microsomal treatment	NADPH-cytochrome *c* reductase (nmoles/mg/min)	Cytochrome b_5 (nmoles/mg)	Cytochrome P-450 (nmoles/mg)	Benzphetamine *N*-demethylase (nmoles formaldehyde/mg/min)	Aryl hydrocarbon hydroxylase (pmoles/mg/min)	Enchanced aryl hydrocarbon hydroxylase (pmoles/min/unit reductase	
						No addition	Plus 1.0 nmole cytochrome P-448
Control	105 ± 2.5	0.28 ± 0.03	0.91 ± 0.02	4.3 ± 0.3	1150 ± 70		
Cumene hydroperoxide	95 ± 5.8	0.34 ± 0.01	0.11 ± 0.02†	0.52 ± 0.03†	9.6 ± 0.50†	0.19 ± 0.012	9.5 ± 0.51
NADPH, ATP, $Fe(II)SO_4$	132 ± 4.5†‡	0.50 ± 0.01†§	0.54 ± 0.06†§	0.86 ± 0.14†	33.8 ± 3.4†§	0.17 ± 0.012	13.0 ± 0.73‡

* Microsomes from male rats were incubated with either cumene hydroperoxide or NADPH, ATP and ferrous sulfate as described in Materials and Methods. The microsomes were recentrifuged, resuspended and assayed for cytochrome b_5, cytochrome P-450, NADPH-cytochrome *c* reductase, benzphetamine *N*-demethylase and aryl hydrocarbon hydroxylase. Additionally, cumene hydroperoxide and NADPH-pretreated microsomes (0.27 mg protein, 28.6 nmoles/min reductase; 0.34 mg protein, 42.8 nmoles/min reductase, respectively) were incubated with 1.0 nmole cytochrome P-448 at 37° for 30 min and the increased rate of aryl hydrocarbon hydroxylase activity was determined. Values are means ± S.E. of three or more samples with the levels of statistical significance indicated.

† Statistical significance vs control, $P < 0.01$.

‡ Statistical significance vs cumene hydroperoxide treatment, $P < 0.01$.

§ Statistical significance vs cumene hydroperoxide treatment, $P < 0.001$.

1.0 nmole of cytochrome P-448, microsomes pretreated with either NADPH or CHP exhibited increases in AHH activity of 76- and 50-fold, respectively. The large increase in AHH activity observed when cytochrome P-448 was incubated with NADPH, ATP and ferrous sulfate-treated microsomes was not due to the EDTA contained in the cytochrome P-448 buffer solution. The constituents of this buffer, alone or in combination, did not increase the AHH activity of the NADPH-treated microsomes.

DISCUSSION

Jeffery *et al.* [19] showed that LAHP had no effect on hepatic microsomal cytochrome b_5 or NADPH-cytochrome *c* reductase while greatly decreasing cytochrome P-450 and P-420 content and both aniline hydroxylase and ethylmorphine *N*-demethylase activities. Yang [1] has also shown that LAHP-treated hepatic microsomes have a greatly decreased cytochrome P-450 content with only small losses in NADPH-cytochrome *c* activity. This study shows that CHP and LAHP have similar effects on the rat hepatic microsomal mono-oxygenase system and confirms the observations of Hrycay and O'Brien [21] who showed that CHP could destroy hepatic cytochrome P-450. The commercially available compound, cumene hydroperoxide, decreases rat microsomal cytochrome P-450, benzphetamine *N*-demethylase and AHH activity, while not altering cytochrome b_5 or hepatic NADPH-cytochrome *c* reductase (Table 1). CHP treatment does, however, lower renal microsomal NADPH-cytochrome *c* reductase activity by about one half.

Both LAHP and CHP cause lipid peroxidation [30]. The oxygen uptake of egg yolk lecithin incubated with cumene hydroperoxide and either cytochrome P-450, hematin or cytochrome b_5 was 495, 300 and 35 nmoles O_2/min/nmole of heme [30]. This lipid peroxidation mechanism may partially explain the observation (Table 1) that CHP causes the greatest decrease in cytochrome P-450 content and MFO enzyme activity in tissues with the highest cytochrome P-450 content, such as hepatic microsomes from male rats.

By use of ultracentrifugation to separate unbound exogenous hemoprotein from hepatic microsomal membranes, it has been shown that both cytochrome P-450 [3] and cytochrome P-448 [2] incorporate into the microsomal membrane during incubation at 37°. By means of Sepharose 4B column chromatography to separate unbound hemoproteins from microsomes, Yang has demonstrated the incorporation of cytochrome P-450 [1] and cytochrome P-448 [2] into hepatic microsomes.

Yang [1] and also Yang and Strickhart [2] have shown that the exogenous cytochrome P-448 incorporated into the microsomes is enzymatically active in accepting electrons from endogenous NADPH-cytochrome *c* reductase [1] and in hydroxylating benzo[*a*]pyrene [1, 2]. However, positive enzymatic evidence of cytochrome P-450 incorporation into microsomal membrane has been more difficult to demonstrate. Miwa *et al.* [3] incubated male liver microsomes with a 30- to 40-fold excess of exogenous cytochrome P-450 and found that the cytochrome P-450 content and benzphetamine *N*-demethylase activity were increased by only 80 and 40 per cent, respectively. In similar experiments, despite a 50 per cent increase in cytochrome P-450 content of hepatic microsomes of phenobarbital-pretreated rats incubated with exogenous cytochrome P-450, no increase in benzphetamine *N*-demethylase activity was found [3]. Yang [1] incubated up to 7.0 nmoles of cytochrome P-450 with control, sonicated and LAHP-pretreated microsomes and observed 0-, 2- and 12-fold enhancement in benzphetamine *N*-demethylase activity. Incubation of cytochrome P-448 with either control or hydroperoxide-treated microsomes increases AHH activity more dramatically than incubation of cytochrome P-450 enhances benzphetamine *N*-demethylase activity.

The studies reported here show that CHP pretreatment may be used to reduce the endogenous microsomal cytochrome P-450 content and MFO activity prior to incorporation of enzymatically active hemoproteins such as cytochrome P-448. Incubation of 4.0 nmoles of cytochrome P-448 with untreated microsomes from adult male rats (200–275 g) did not significantly increase AHH activity. This is in contrast to the results of Yang [1] who demonstrated a 66 per cent increase by incubating hepatic microsomes from immature (50–150 g) male rats with cytochrome P-448. The lower AHH activity of younger rats may account for this difference. When CHP-pretreated hepatic microsomes from female and male rats were incubated with exogenous cytochrome P-488, AHH activity was elevated 31- and 53-fold. The enzymatic ability of incorporated cytochrome P-448 to hydroxylate benzo[*a*]pyrene was evident in untreated hepatic microsomes from female (4.2-fold increase) and 21-day fetal rats (12-fold increase).

The enhanced AHH rates for CHP-treated microsomes divided by the values for untreated microsomes were 1.46, 0.42, 0.13 and 0.12 for the female liver, fetal liver, male kidney and female kidney, respectively. Since CHP lowers endogenous cytochrome P-450 concentration, lessened competition for reductase electrons may explain the increased relative enhanced AHH rate for female liver microsomes. The decreased relative enhanced AHH rate for fetal liver might be due to lessened cytochrome P-448 incorporation into the microsomal membranes or less efficient coupling with endogenous NADPH-cytochrome *c* reductase caused by CHP-induced membrane damage. The greatly decreased relative enhanced AHH rate in kidney microsomes suggests reduced cytochrome P-448 incorporation and/or less efficient coupling with CHP-depleted endogenous NADPH-cytochrome *c* reductase. Fetal microsomal cytochrome P-450 and MFO enzyme activity are much lower [34] than in the adult. Fetal rat liver MFO enzyme activities are not induced by phenobarbital [50, 51] but AHH activity can be induced by polycylic hydrocarbons [52–54] or TCDD [55]. The results of this study suggest that 21-day fetal AHH activity is low due to the low cytochrome P-450 (P-488 form) content. The lipid and NADPH-cytochrome *c* reductase present in fetal liver microsomes are capable of supporting 12-fold higher rates

of AHH activity after incubation with 1.0 nmoles of exogenous cytochrome P-448.

The increases of AHH activity due to incorporation of enzymatically active cytochrome P-448 are best demonstrated with microsomal membranes high in NADPH-cytochrome *c* reductase and low in endogenous AHH activity. Female rat hepatic and renal microsomes and hepatic microsomes from 21-day fetal liver are the most attractive choices for cytochrome P-448 and microsomes interaction studies using AHH as an end point. Female rat liver microsomes are particularly attractive because they have three times as much NADPH-cytochrome *c* reductase activity as do renal microsomes. Hepatic microsomes from CHP or NADPH-treated male rat liver also have high NADPH-cytochrome *c* reductase and low AHH activities, but suffer from the complication of lipid damage which occurs while denaturing the endogenous cytochrome P-450.

A comparison of CHP treatment with NADPH-dependent lipid peroxidation shows both similarities and differences. Cytochrome P-450 content, benzphetamine *N*-demethylase activity and AHH activity are decreased to a greater extent by cumene hydroperoxide than by NADPH. NADPH decreases microsomal protein values more than does cumene hydroperoxide, and thus causes increases in the specific NADPH-cytochrome *c* reductase and in the cytochrome b_5 content per mg microsomal protein.

The greater increase in AHH activity observed after incubation of NADPH- as compared to CHP-treated microsomes with cytochrome P-448 (37 per cent) may be partially due to the differences in specific NADPH-cytochrome *c* reductase activity between these two samples (18 per cent). Alteration of the microsomal membrane structure via NADPH-induced lipid peroxidation may change the amount of exogenous cytochrome P-448 incorporated into the membrane. Sonicated rat liver microsomes incubated with cytochrome P-450 have exhibited a higher enhanced benzphetamine *N*-demethylase rate than did unsonicated controls [1]. The use of organic hydroperoxides rather than NADPH-dependent lipd peroxidation to lower endogenous cytochrome P-450 is nevertheless preferable for two reasons. Glutathione may be used to stop the destruction of cytochrome P-450 by either CHP [21] or LAHP [19]. With NADPH, ATP and ferrous sulfate, it is more difficult to stop lipid peroxidation and to remove the three agents. In addition, cumene hydroperoxide is both more specific and efficient than NADPH treatment in reducing cytochrome P-450 and MFO enzyme activities while not altering NADPH-cytochrome *c* reductase activity or cytochrome b_2 content. Hydroperoxides also destroy partially purified cytochrome P-450 *in vitro* [27].

In conclusion, microsomes low in endogenous AHH activity were incubated with partially purified cytochrome P-488 and the resulting increases in AHH activity were determined. Organ, sex and ontogenic differences in microsomal AHH activity permit a more sensitive demonstration of the incorporation of enzymatically active exogenous cytochrome P-448 than is possible using hepatic microsomes obtained from adult male rats. Alternatively, cumene hydroperoxide may be used to reduce microsomal cytochrome P-450 and AHH activity prior to the incorporation of exogenous cytochrome P-488. In cytochrome P-488 and microsomes enzymatic interaction studies, sensitive detection of enhanced catalyic rate is a desirable property as many current cytochrome P-488 purification procedures have low yields. Enzymatic interaction studies using control or chemically modified microsomes and exogenous cytochromes of the P-450 class may be useful in providing information about the characteristics of different microsomal membranes such as their ability to incorporate exogenous cytochromes and the efficiency with which these incorporated cytochromes couple with endogenous electron transport proteins.

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