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MONOCLONAL ANTIBODY CHARACTERIZATION OF NADH- AND NADPH-DEPENDENT HYDROXYLATION OF BENZO(A)-PYRENE IN LIVER MICROSOMES FROM 5,6-BENZOFLAVONE-INDUCED C57Bl/6 MICE

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Monoclonal antibodies (MAb 1-7-1) directed against the isoenzymes of rat liver cytochrome P-450 induced by methylcholanthrene, inhibited benzo(a)pyrene hydroxylase less strongly at low (0.04 mM) than at high (2 mM) NADH concentration. Inhibition of NADPH dependent hydroxylation was the same irrespective of NADPH concentrations and corresponded to that observed at high NADH concentration. The same was also the h.p.l.c. pattern of the reaction products of benzo(a)pyrene hydroxylation supported by high concentration of both coenzymes. It is postulated that different cytochrome P-450 isoenzymes participate in benzo(a)pyrene hydroxylation, whereas the second one acts at high concentration of both NADH and NADPH.

Microsomes contain both NADPH- and NADH-linked electron transport systems. The first one consists of the flavoprotein NADPH-cytochrome P-450 reductase and cytochrome P-450 isoenzymes. In this system, NADPH-cytochrome P-450 reductase transfers electrons from NADPH to cytochrome P-450, which binds various endogenous and xenobiotic substrates metabolized by mixed-function type oxidases (monooxygenases). The second system consists of NADH-cytochrome b_5 reductase plus cytochrome b_5 and provides electrons for a variety of microsomal reactions, including stearyl-CoA desaturation (for review see [1]). In recent years, it has become apparent that these two systems do not function independently. Based on the synergistic effect of NADH on the NADPH-dependent reactions, Hildebrandt & Estabrook [2] first proposed that cytochrome b_5 is also involved in the NADPH-dependent reactions of liver microsomes and that the second of two electrons required for the overall monooxygenase reaction, is supplied to

cytochrome P-450 *via* cytochrome b_5. It has been found in more recent studies with reconstituted systems that the influence of cytochrome b_5 on the cytochrome P-450-mediated reactions varies for different isoenzymes of cytochrome P-450 [3, 4]. In mouse liver microsomes, NADH can alone sufficiently provide electrons needed in benzo(a)pyrene (BP) hydroxylation, although this reaction is only one-tenth of the rate observed for the NADPH-mediated reaction [5, 6]. There are also differences between kinetics of BP hydroxylase activity at various concentrations of NADPH as compared to NADH [7]. The aim of this study was to assess the contribution of cytochrome P-450 isoenzymes recognized by monoclonal antibody MAb 1-7-1 in NADH- and NADPH-dependent hydroxylations of BP.

MATERIALS AND METHODS

Reagents. NADH, NADPH and benzo(a)pyrene were from Sigma Chemical Co. Tritium labeled benzo(a)pyrene (G-[^{3}H]BP) from Amersham International plc. was repurified by h.p.l.c. shortly before the experiments. All other chemicals were of the highest commercial purity available.

Antibodies. The monoclonal antibody towards two methylcholanthrene--induced isoenzymes of rat liver cytochrome P-450 was from the clone 1-7-1 (hereafter referred to as "MAb 1-7-1") [8, 9]. Ascites fluid from hybrid cells formed from myeloma cells and spleen cells from unimmunized mice (NBS 1-48-5) containing nonspecific IgG [8] was used as control. Both MAb 1-7-1 and NBS 1-48-5 were a generous gift from Prof. H. V. Gelboin.

Animals. The experiments were performed on 6-10 week old male C57Bl/6 mice. For the induction of BP hydroxylase (AHH), 5,6-benzoflavone (5,6-BF) was injected intraperitoneally (80 mg/kg body weight as 4 mM solution in sunflower oil). Animals were killed by dislocation of spinal cord 40 h after 5,6-BF treatment.

Preparation of microsomes. Livers from 5-8 mice were used for one preparation of microsomes. After killing of animals livers were quickly removed, dissected free of the gallbladder and adhering tissues and placed in ice-cold 0.1 M sodium/potassium phosphate buffer, pH 7.4. All subsequent manipulations were conducted at 0-4°C; livers were minced, washed, and suspended in three volumes of the buffer. Homogenization was carried out with glass-teflon homogenizer. The microsomes were isolated by the standard ultracentrifugation technique, resuspended in a small volume of the buffer containing 30% glycerol and stored at −80°C. Protein content in microsomes was estimated by the method of Lowry *et al.* [10] using crystalline bovine serum albumin as a standard.

Determination of AHH activity. The activity of AHH was assessed by measuring formation of the fluorescent phenol derivatives of BP. The incubation mixture contained: 50 mM potassium phosphate buffer, pH 7.25;

3 mM KCl; 3 mM $MgCl_2$; 40 μM or 2000 μM NADH (NADPH); 80 μM BP; 0.2 mg of microsomal protein/ml, and MAb 1-7-1 or NBS 1-48-5. The ratio of antibody protein to microsomal protein was 2:5. The temperature was equilibrated to 37°C. The reaction was started by the addition of NADH or NADPH. The extraction of BP-phenols was performed according to Nebert & Gelboin [11]. Fluorescence of BP-phenols was measured at emission wavelength of 520 nm, using excitation wavelength of 392 nm, on Perkin-Elmer 650-10S spectrophotofluorimeter. To obtain the initial velocity of BP hydroxylation, the production of BP-phenols was estimated at 30 second intervals during incubation lasting up to 3 min. The progress curve was drawn from several measurement points and a tangent at the origin of the curve was used to determine the initial velocity at each concentration of NAD(P)H. The curves were linear for 2.5 min at the 40 μM concentration of coenzymes.

h.p.l.c. *Analysis of [^{3}H]BP metabolites.* Microsomes were incubated with tritium labeled BP (specific activity 50 mCi/mmol) for 10 min at 37°C. Incubation mixture was the same as in determination of AHH activity. NAD(P)H concentration was 2 mM; concentration of microsomal protein 1 mg/ml. BP metabolites were extracted from incubation mixtures by ethyl acetate. The organic layer was dried over sodium sulfate, and evaporated under a stream of nitrogen. BP metabolites were separated by h.p.l.c. The column (Lichrosorb RP 18) was eluted with a linear gradient of 60% methanol in water to 100% methanol in 45 min at a flow rate of 0.8 ml/min. Radioactivity of fractions was measured. Metabolites were identified either by comparing their retention times to those of standards or by comparing the absorbance spectra of the eluted peaks with literature data [12].

Table 1

The effect of MAb 1-7-1, the monoclonal antibody against 3-methylcholanthrene-induced rat liver cytochrome P-450, on the NADH- and NADPH-supported AHH activity in the 5,6-benzoflavone-induced mouse liver microsomes. The data represent mean results from 4 experiments ± SD

Concentration of coenzymes		AHH activity (pM 3-OH-BP/min per mg)		Inhibition (%)
		Control	MAb 1-7-1	
NADH	40 μM	21.2± 3.3	18.3± 0.4	13.7
	2000 μM	207.1±14.9	59.4± 3.2	71.3
NADPH	40 μM	1298.0±73.2	375.1±46.3	71.1
	2000 μM	1426.6±68.5	384.4±44.8	73.0

RESULTS

Effects of MAb 1-7-1 on AHH activity

Table 1 presents the inhibition of cytochrome P-450 catalyzed BP hydroxylation by monoclonal antibodies. MAb 1-7-1 inhibition of AHH activity supported by low concentration of NADH was considerably smaller than that supported by high concentration of this coenzyme. There were no evident differences between the extent of inhibition of AHH activity supported by high concentration of NADH as compared to NADPH.

h.p.l.c. Separation of BP-metabolites

h.p.l.c. Patterns of BP-metabolites are similar in NADH- and NADPH-supported reactions (Fig. 1), although the amount of BP-metabolites produced in the NADH-supported reaction is much lower as compared to the NADPH-supported reaction (Table 2). The proportion of 9,10-diol to the other metabolites was higher in the NADH- than in NADPH-supported metabolism of BP (Table 2).

DISCUSSION

The effects of MAb 1-7-1 on BP hydroxylase activity supported by low and high concentrations of NADH and NADPH were investigated. Concentrations of coenzymes were those at which the kinetics of BP hydroxylation in liver microsomes of 5,6-BF-treated C57Bl/6 mice with respect to NADH was biphasic [7]. Biphasic kinetics of the NADH-supported (but not

Table 2

The effect of NADH and NADPH on production of different [^{3}H]BP metabolites separated by h.p.l.c. (see Materials and methods).

The data represent mean results from 3 experiments ± SD

	BP metabolites (nmol) produced in reaction supported by		% of activity NADH/NADPH
	NADPH	NADH	
3-OH-BP	13.84 ± 1.52	2.37 ± 0.12	17.12
9-OH-BP	7.65 ± 0.50	1.64 ± 0.56	21.44
Quinones	4.31 ± 0.60	1.15 ± 0.24	26.68
7,8- and 4,5-diol	5.74 ± 0.76	1.47 ± 0.02	25.61
9,10-Diol	0.89 ± 0.13	0.57 ± 0.04	64.04
Polar metabolites	0.51 ± 0.32	0.20 ± 0.16	39.22

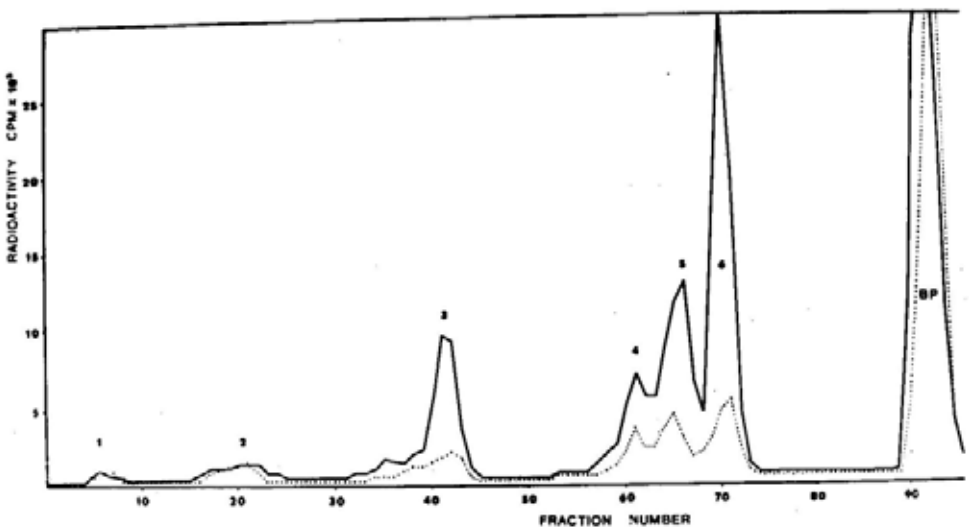

Fig. 1. H.p.l.c. pattern of [^{3}H]BP metabolites obtained during NADH-supported (dotted line) and NADPH-supported (continuous line) reactions. 1. Polar metabolites; 2. 9,10-diol; 3. 7,8- and 4,5-diol; 4. quinones; 5. 9-OH-BP; 6. 3-OH-BP

NADPH-supported) cytochrome P-450-dependent reduction of indicine *N*-oxide in rat liver microsomes [13], and similar cinnamic acid 4-hydroxylation in higher plant microsomes [14] suggest that biphasicity is an universal phenomenon connected with the cytochrome b_5 pathway. Differential MAb 1-7-1 inhibition of AHH activity at low and high concentrations of NADH (Table 1) indicates that various cytochrome P-450 isoenzymes are responsible for AHH activity observed at each phase of biphasic kinetics. On the other hand, the same cytochrome isoenzymes take part in AHH activity supported by high concentrations of both NADH and NADPH. This conclusion is in concordance with an earlier observations that 5,6-BF induction of the NADH-dependent AHH activity in C57Bl/6 mice depends on the concentrations of this coenzyme; there was no induction at the lowest concentration, whereas at the highest concentration of NADH the induction was equal to that of NADPH-dependent activity [7]. It might be suggested that at the low concentration of NADH, electrons are supplied by the cytochrome b_5 pathway predominantly to those isoenzymes of cytochrome P-450 which are characterized by the highest affinity to cytochrome b_5, and are neither induced by 5,6-BF nor recognized by MAb 1-7-1. Those isoenzymes must compete with other cytochrome b_5-dependent reactions using endogenous substrates (e.g. desaturation of fatty acids). We noted the high consumption of NADH in mouse liver microsomes preparation without

addition of BP (not shown). The existence of isoenzymes of cytochrome P-450, having high affinity to cytochrome b_5 has been reported elsewhere [3, 15, 16].

The h.p.l.c. patterns of BP metabolites in NADH- and NADPH-supported BP hydroxylations are qualitatively similar (Fig. 1), however, the amount of metabolites produced in the NADPH-supported reaction is significantly higher (Table 2). The similarity of the h.p.l.c. pattern observed at high concentrations of both coenzymes corresponds to a similar inhibitory effect of MAb 1-7-1 on NADH- and NADPH-dependent AHH activity (Table 1).

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