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ARYLHYDROCARBON HYDROXYLASE ACTIVITY AND CYTOCHROME *P*-450 IN HUMAN TISSUES

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Summary

The stability and distribution of arylhydrocarbon hydroxylase activity in four human tissues has been examined. Two tissues, liver and lung, were obtained from autopsy samples while lymphocytes and placenta were obtained from cell lines and donors Marked differences in arylhydrocarbon hydroxylase activity were observed between tissues and individuals, with liver being the richest source. Activity in all tissues was stable at 4°C for 24 h, but freeze-thawing markedly reduced hydroxylase activity in liver. Using gel exclusion chromatography, the molecular weight of a non-dissociated form of arylhydrocarbon hydroxylase was estimated to be about 400 000. A heme staining band corresponding to a molecular weight of 50 000 was observed after polyacrylamide gel electrophoresis of liver microsomal preparations This appears to be a cytochrome *P*-450 subunit based on correlations between staining intensity and hydroxylase activity in tissues and partially purified preparations examined

Introduction

The mixed function oxygenases are a family of enzymes that metabolize most environmental chemicals, including drugs [1], polycyclic aromatic carcinogens [2], fatty acids [3,4] and steroids [5]. These enzymes are localized in microsomal membranes and are known collectively as the cytochrome *P*-450 proteins. Arylhydrocarbon hydroxylase is part of the *P*-450 complex catalyz-

Abbreviations TEMED, *N,N,N′,N′*-tetramethylethylenediamine, TMBZ, 3,3′,5,5′-tetramethylbenzidine

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ing the first step in the metabolism of benzopyrene and other polycyclic aromatic hydrocarbons [6,7]. Arylhydrocarbon hydroxylase is of particular interest because of the possible correlation between its inducibility and susceptibility to polycyclic aromatic hydrocarbon induced tumors in animals [8] and possibly in humans [9—12].

Studies of human cytochrome *P*-450 proteins have been hampered by the limited availability of tissue and by the low tissue levels of cytochrome *P*-450. Alvares et al [13] found an average of 250 pmol *P*-450/mg microsomal protein in three human livers. In contrast, mouse and rat liver contain about 800 pmol *P*-450/mg microsomal protein [14,15]. Mixed function oxygenase activities have been measured in human liver [16], placenta [17] and breast [18] for a number of substrates including benzo[*a*]pyrene. The ability of human tissue to metabolize various drug and carcinogen substrates suggests the presence of multiple forms of cytochrome *P*-450 However, the quantitative and qualitative nature of these cytochrome *P*-450 proteins and their role in the biochemistry and genetics of drug and carcinogen metabolism remains to be elucidated.

Multiple forms of cytochrome *P*-450 exist in tissues of laboratory animals [14,15] and there is evidence for multiple forms of *P*-450 in human liver. Several forms of human liver cytochrome *P*-450 have been isolated to apparent homogeneity as judged by SDS-polyacrylamide gel electrophoresis [16]. Subunit molecular weights are estimated to be 45 000 [19] and 53 000 and 55 500 [16]. These proteins catalyze a variety of oxygenase reactions including benzo[*a*]pyrene hydroxylation [16,19,20].

Previous work in our laboratories [17,21] showed that arylhydrocarbon hydroxylase activity is induced in human placenta by smoking and in lymphocytes by treatment with 3-methylcholanthrene In this paper we attempt to utilize this induction behavior to locate *P*-450 proteins on SDS-polyacrylamide gels. We examine microsomal proteins from liver, lung, placenta and lymphocytes and compare gel electrophoresis patterns and arylhydrocarbon hydroxylase activities in the four tissues. Also presented are molecular weight estimates of a *P*-450 subunit identified after gel electrophoresis by heme staining and of a non-dissociated form of *P*-450 determined by gel exclusion chromatography.

Materials and Methods

Materials Acrylamide, bisacrylamide, TEMED and ammonium persulfate were from Bio-Rad, Rockville Center, NY. Ovalbumin, carbonic anhydrase and β-lactalbumin were from Pharmacia. SDS, crystalline bovine serum albumin, cytochrome *c*, sodium deoxycholate, TMBZ, hydrogen peroxide, trinitrobenzene sulfonic acid, Tris and EDTA were from Sigma. Ultrogel AcA-34 was from LKB.

Tissue sources Samples of human liver were obtained at autopsy from three subjects, one male and two females (66—73-years-old). Deaths were due to suffocation for the male (LV1) and adenocarcinoma of the cecum (LV2) and abdominal carcinoma (LV3) for the females. There were no apparent metastases to the liver for the cancer patients. The autopsies were performed within 4 h after death and the liver samples were frozen at —70° C. A sample of human lung (LG1) obtained from the male subject at autopsy, was also frozen at

—70° C. Human placentas were obtained and microsomes prepared as described by Vaught et al. [17]. Human lymphocytes were taken from a long term cell line (RPMI-1788) and from buffy coats of healthy plasmapheresis donors. Lymphocytes were cultured, and some were induced with 3-methylcholanthrene and harvested according to Paigen et al. [21] The cells were frozen at —70° C Microsomes from rat livers were a generous gift from Dr. Hira Gurtoo (Roswell Park Memorial Institute).

Microsomal membrane preparations Microsomes from human liver and lung were prepared at 4° C. 5—10 g tissues were thawed, diced and homogenized by hand, using a ground-glass homogenizer in 3 vol. 0.15 M KCl. The homogenate was centrifuged at 9500 × *g* for 15 min and the pellet reconstituted with 3 vol. 0.15 M KCl This solution was again centrifuged at low speed and the two supernatants were combined and centrifuged at 16 000 × *g* for 20 min. The supernatant was centrifuged at 100 000 × *g* for 1 h and the resulting pellet was resuspended in 8 ml 0.15 M KCl and centrifuged again at 100 000 × *g* for 1 h The pellet was resuspended in 0.01 M Tris-acetate (pH 7.4)/0.15 M KCl/0.1 mM EDTA/10% glycerol and stored at —70° C or used immediately In some cases, the pellet was resuspended in 4 ml 0 15 M KCl and layered on a sucrose gradient consisting of 1 ml 2.0 M sucrose, 3.5 ml 1.6 M sucrose, 3 5 ml 1.4 M sucrose, and centrifuged for 1 h at 40 000 × *g* Fractions were brought to 4 ml with 0 15 M KCl and centrifuged at 100 000 × *g* for 1 h. Pellets were resuspendended in the Tris-acetate buffer to 4 ml and used immediately or stored frozen at —70° C.

Preparation of microsomes derived from human placentas was as described by Vaught et al. [17].

Arylhydrocarbon hydroxylase activity. Lymphocyte and placental microsomes were assayed for arylhydrocarbon hydroxylase activity according to Gurtoo et al. [22] and Vaught et al [17], respectively. Liver and lung microsomes were assayed for activity by modification of the lymphocyte assay. To achieve optimum assay conditions in liver and lung microsomes, the time of incubation was increased from 30 min to 1 h and the pH altered from pH 8.6, for lymphocytes and placenta, to pH 7 6. Specific activity is expressed as pmol 3-hydroxybenzo[*a*]pyrene formed/min per mg microsomal protein. Protein was determined as described by Lowry et al [23] using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis Polyacrylamide gel electrophoresis was performed in the presence of SDS using slab gel electrophoresis according to Laemmli [24] with slight modifications. Protein fractions at a final concentration of 1.6 mg/ml were first treated for 30 min at 70° C with 0.06 M Tris-HCl (pH 6.8)/2% SDS/10% glycerol/5% 2-mercaptoethanol/0 01% bromophenol blue The slab separating gel contained 10% acrylamide, and the stacking gel contained 3% acrylamide. Electrophoresis was carried out at 20° C for 8 h at 20 mA/gel. Proteins were detected by staining overnight in 0.25% Coomassie brillant blue R, followed by diffusion destaining in 50% methanol/7% acetic acid. The molecular weights of microsomal proteins were estimated using the protein standards *Escherichia coli* β-galactosidase (M_r = 135 000), bovine serum albumin (M_r = 68 000), glutamate dehydrogenase (M_r = 53 000), ovalbumin (M_r = 45 000), aldolase (M_r = 40 000), carbonic anhydrase (M_r = 32 000), β-

lactalbumin (M_r = 18 000) and horse cytochrome *c* (M_r = 11 000).

Heme staining. Polyacrylamide slab gels of microsomal proteins were subjected to staining with TMBZ in order to visualize hemoprotein components of microsomal samples, according to Thomas et al. [25]. In some cases the slab gels were first washed in 0.02 M Tris-HCl (pH 7.5)/50% methanol for 1 h. The stained bands, which disappeared within an hour, were marked with small holes on either side of the band and stained for protein, according to Thomas et al. [25].

Gel exclusion chromatography Gel filtration studies were carried out at 4°C and a flow rate of 6 ml/h using Ultrogel AcA-34 in a column with dimensions 1.6 × 80 cm. The column was equilibrated with 0 1 M Tris-acetate (pH 7.4)/ 0 1 M KCl/1 mM EDTA/1 mM 2-mercaptoethanol/5—10% glycerol.

Results and Discussion

Stability of human microsomal proteins The stability of human liver microsomal proteins and of arylhydrocarbon hydroxylase activity (in 0.01 M Tris-HCl/0 15 M KCl/1 mM EDTA, pH 7.6) was determined under a variety of conditions Thawing of liver microsomes which had been frozen overnight resulted in about 70% loss of arylhydrocarbon hydroxylase activity, but there was no significant proteolytic degradation of the major proteins as judged by patterns of proteins separated by polyacrylamide gel electrophoresis in SDS. After the initial loss of activity resulting from freezing, the activity of liver microsomes stored frozen at —70°C remained constant for at least 6 months. Concentrations of protein ranging from 0.5—50.0 mg/ml yielded similar results At 4°C the arylhydrocarbon hydroxylase activity of microsomes remained constant for at least 24 h, but at room temperature the bulk of the activity was lost within 3—4 h.

Comparison of human and rat liver microsomal proteins In laboratory animals, including rat [15,26], rabbit [27] and mouse [14], cytochrome *P*-450

TABLE I

ARYLHYDROCARBON HYDROXYLASE ACTIVITY IN MICROSOMES FROM HUMAN TISSUES

Values for LV2 and lung are based on single preparations

Tissue	Specific activity (pmol/min per mg) (±S D)
Liver - LV1	0 49 ± 0 13
Liver - LV2	8 21
Liver - LV3	9 50 ± 3 75
Lung - LG1	0 32
Lymphocyte - uninduced	0 03 *
Lymphocyte - induced	0 08
Placenta - non-smoker	0 06 **
Placenta - smoker (2 pks/day)	4 55

* From Ref 21
** From Ref 17

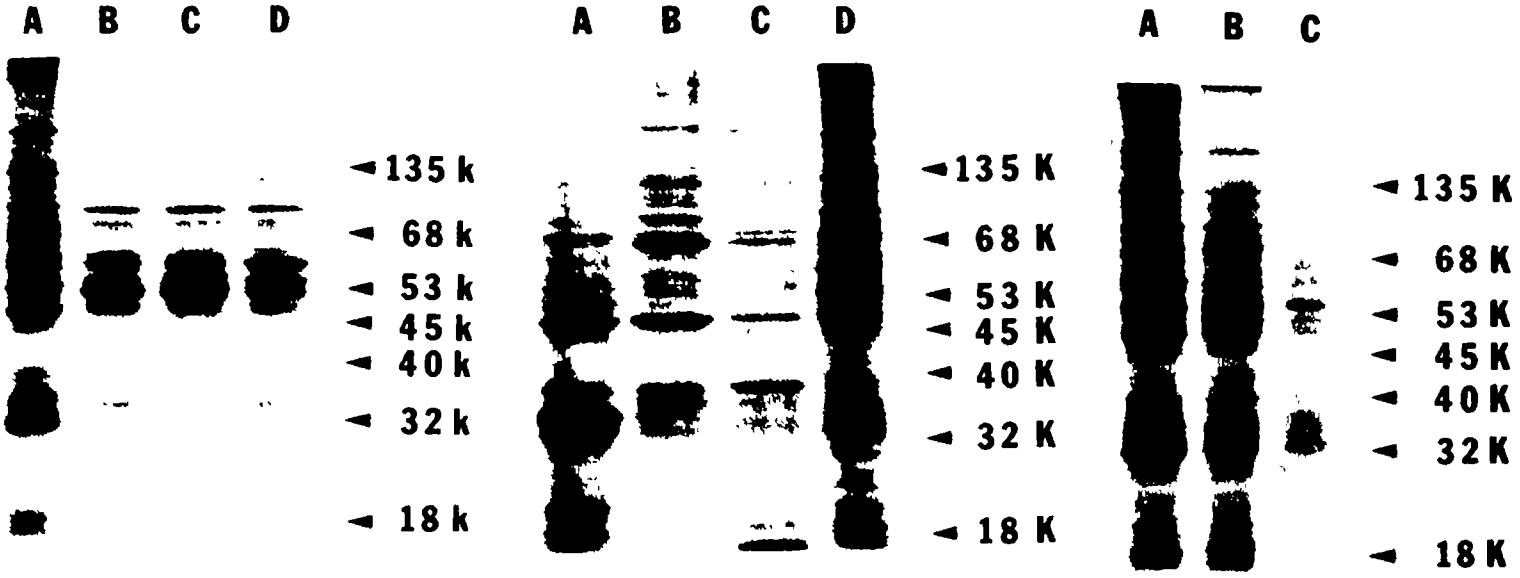

Fig 1 Microsomal proteins from human and rat liver Microsomes (100 μg total protein) were subjected to polyacrylamide gel electrophoresis in the presence of SDS The gel was stained for protein using Coomassie blue The human liver (A) is from a male (LV1) Rat livers were obtained from animals which were uninduced (B) or induced with phenobarbitol (C) or 3-methylcholanthrene (D) [22] The arylhydrocarbon hydroxylase specific activities for the samples are 0 49 (LV1) and about 1770 (rat liver) pmol 3-hydroxybenzo[*a*]pyrene/min per mg microsomal protein Molecular weight standards are indicated by arrows

Fig 2 Microsomal proteins isolated from several human tissues Microsomes were subjected to polyacrylamide gel electrophoresis in the presence of SDS, and the gel was stained for protein using Coomassie blue Molecular weight standards are shown by arrows Lung and liver microsomes are from a heavy smoker Lymphocyte microsomes are from donor cells and were induced with 3-methylcholanthrene [10] Specific activities are given in Table I (A) lung, (B) placenta, (C) lymphocyte, (D) liver

Fig 3 Human liver microsomal proteins from several individuals Microsomes were subjected to polyacrylamide gel electrophoresis in the presence of SDS, and the gel was stained for protein using Coomassie blue Slots (A) and (B) each contained about 100 μg protein while slot (C) contained about 50 μg protein Molecular weight standards are indicated by arrows Specific activities are given in Table I (A) LV1, (B) LV2, (C) LV3

and *P*-448 proteins have been found to have molecular weights ranging from about 47 000 to 60 000. In rat liver microsomes, these are the most prominent proteins, particularly after induction with phenobarbitol or 3-methylcholanthrene. When rat liver microsomal proteins are subjected to electrophoresis in the presence of SDS, the *P*-450 and *P*-448 proteins are seen to migrate to positions corresponding to molecular weights of 48 000 and 53 000, respectively [15,26]. Human liver microsomal proteins do not appear to co-migrate with the rat cytochrome *P*-450 or *P*-448 proteins (Fig 1) The level of proteins in the 45 000—60 000 molecular weight range, after electrophoresis in SDS, was much lower for microsomes from human liver than rat liver. Moreover, the intensity of heme staining of human microsomal proteins in this molecular weight range was considerably less than for rat microsomal proteins. This is consistent with the apparently lower levels of *P*-450 proteins in human liver than in rat liver, as judged by arylhydrocarbon hydroxylase activity measurements and *P*-450 absorption measurements for human liver [28]

Differences in human microsomal proteins and arylhydrocarbon hydroxylase activities between tissues and individuals Arylhydrocarbon hydroxylase activities and gel electrophoresis patterns for human liver, lung, placenta and lymphocytes are presented in Table I and Fig. 2. Liver is the richest source of activity. The patterns of proteins observed after electrophoresis showed striking differences; in particular, placenta and lymphocytes had a much lower concentration of microsomal proteins in the 45 000—60 000 molecular weight range than liver and lung.

Differences in both liver arylhydrocarbon hydroxylase activities and gel electrophoresis patterns were observed for the different individuals tested. One of the livers (LV1) had considerably lower activity than the others (LV2, LV3) as well as reduced levels of a heme-staining band migrating in gel electrophoresis at a position corresponding to 50 000 daltons (Fig. 3). The gel pattern of LV1 microsomes was otherwise quite similar to LV2 and LV3 microsomes. This band may correspond to a cytochrome *P*-450, and it is noteworthy that there is a correspondence between the intensity of this band and the level of arylhydrocarbon hydroxylase activity.

Differences in the arylhydrocarbon hydroxylase activity among the three liver autopsy samples (Table I) could have been due to partial degradation of components of the *P*-450 system. Although this possibility cannot be eliminated, we feel the differences in activity may well result from genetic variations because liver samples were obtained and processing was begun within 4 h of death, and there appeared to be no significant proteolytic degradation of the major proteins as judged by patterns of proteins separated by polyacrylamide gel electrophoresis in SDS. Moreover, differences in arylhydrocarbon hydroxylase activity among individuals have been observed in other human tissues such as placenta [17] and lymphocytes [11]

Induction of human P-450 proteins We attempted to utilize the induction behavior of arylhydrocarbon hydroxylase activity to locate human *P*-450 proteins on SDS-polyacrylamide gels In lymphocytes, as much as a 5-fold induction in activity is observed using 3-methylcholanthrene as inducer [21] In placenta, cigarette smoking during pregnancy induces a variety of enzymes involved in the metabolism of benzo[*a*]pyrene, including arylhydrocarbon hy-

droxylase which is increased about 100-fold in smoking mothers [17] However, no increases in band intensities were observed on our gels for induced lymphocytes and placenta from smoking mothers using protein and heme staining. This suggests that *P*-450 proteins contributing to hydroxylase activity are present in very low concentrations in these tissues.

Molecular weight estimates for human P-450 proteins Autor et al [29] used gel exclusion chromatography to estimate the molecular weight of non-dissociated cytochrome *P*-450 proteins of the rabbit. We have employed this method to estimate the molecular weight of human *P*-450 proteins. Arylhydrocarbon hydroxylase activity and gel electrophoresis were used to monitor the location of *P*-450 proteins in the resulting fractions. The bulk of the activity was eluted in a volume corresponding to molecular weight of about 400 000 which is close to the 350 000 peak observed for rabbit cytochrome *P*-450 A smaller peak was occasionally observed in fractions corresponding to about 50 000 daltons. When subjected to electrophoresis in SDS, the fraction containing arylhydrocarbon hydroxylase activity was enriched in the 50 000 dalton heme staining band.

Enrichment of this heme staining band was also seen following subcellular fractionation of liver microsomes by sucrose density gradient centrifugation. We routinely observed correspondence between levels of arylhydrocarbon hydroxylase activity in the fractions and the intensity of the heme staining band at 50 000 daltons.

Several lines of evidence suggest that the heme staining band migrating at a position corresponding to 50 000 daltons is in fact cytochrome *P*-450. First, it is in the molecular weight range expected on the basis of studies of other mammalian cytochrome *P*-450 proteins [14,15,26,27]. Second, it copurifies with arylhydrocarbon hydroxylase activity using gel exclusion chromatography. And third, there is a correlation between the levels of hydroxylase activity and the intensity of the electrophoresis band in the various individuals and tissues examined.

Recently, the partial purification of cytochrome *P*-450 from human liver was described [16,19,20]. Subunit molecular weights for *P*-450 proteins were estimated to be 53 000 and 55 500 [16] and 45 000 [19]. Our studies indicate a subunit molecular weight of about 50 000. Whether this descrepancy is a result of methodological differences or to multiple *P*-450 proteins is unknown.

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