Aryl Hydrocarbon Hydroxylase Activity in Human Placenta from Cigarette Smoking and Nonsmoking Women

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SUMMARY

Aryl hydrocarbon hydroxylase activity was determined in the placentas obtained from 97 women at the time of childbirth. Forty-six of the women smoked between 2 and 40 cigarettes daily during the pregnancy, and 51 women were nonsmokers. Significantly higher levels (P < 0.001) of placental aryl hydrocarbon hydroxylase were found in women with a history of cigarette smoking.

INTRODUCTION

Microsomal aryl hydrocarbon hydroxylase is known to be present and inducible by polycyclic hydrocarbons in a variety of tissues of many species in vivo, in explants of rat lung in organ culture, and in mammalian cell cultures. This enzyme system may be important in understanding carcinogenesis, drug action, and steroid metabolism. Polycyclic hydrocarbons are present as environmental or food contaminants as well as in cigarette smoke and are commonly ingested and inhaled by the human population. Some of the cancer incidence in humans may be the result of exposure to these substances. The level and nature of the enzyme system metabolizing polycyclic hydrocarbons would be expected to play a key role in polycyclic hydrocarbon carcinogenesis. Similarly, the levels of aryl hydrocarbon hydroxylase and related microsomal enzymes are relevant to microsomal drug and steroid metabolism.

Welch and coworkers have reported the presence of benzyrene hydroxylase in all of the placentas from 17 women smokers and in none of the placentas from 17 nonsmokers. The results of our study indicate a high but not absolute correlation between a history of smoking and an increased level of placental aryl hydrocarbon hydroxylase activity.

MATERIALS AND METHODS

Materials. Reduced nicotinamide adenine dinucleotide (NADPH) was purchased from Calbiochem or Sigma. Benzo[a]pyrene, obtained from Eastman, was purified by recrystallization. Purified 3-hydroxybenzo[a]pyrene was a gift from Dr. Hans Falk. Fresh hemoglobin was donated by Miss J. Pastewka. Bovine serum albumin was purchased from Armour Pharmaceutical Company. An Aminco-Bowman spectrophotofluorometer (Model 4-8202) was used.

Collection of Samples. Normal-appearing segments of placenta were obtained from 97 women delivering at the National Naval Medical Center between March and September 1968. The study was carried out on a double-blind basis; the samples were assayed for aryl hydrocarbon hydroxylase activity at the National Cancer Institute, and histories of cigarette smoking were kept by the Department of Obstetrics and Gynecology, National Naval Medical Center. The clinical histories included frequency of smoking during early gestation, the final week, the final three days, and the day of delivery. Other factors recorded were age, race, and drugs administered during pregnancy, labor, and delivery.

Immediately after delivery, the placenta was frozen at −20°C. The hydroxylase activity in 59 placentas was determined within 24 hours of delivery and in 38 placentas between 2 and 7 days after delivery. The placenta was usually divided (while frozen) into samples ranging in size from 2 to 8 grams. One or
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more of these samples was then assayed for hydroxylase activity, while the remainder was stored at -20°C. The half-life of placenta aryl hydrocarbon hydroxylase was determined with samples stored at 0, -20, and -70°C; at each temperature, samples from 2 to 4 different placentas were studied. In several cases, the hydroxylase activity of a 35-gram sample was compared to that of a 5-gram sample. Homogenization of the placental samples in 0.25 M sucrose-0.05 M Tris chloride buffer (pH 7.5) was carried out immediately prior to the enzyme assay; a tight-fitting Potter-Elvehjem glass-glass homogenizer was used.

**Enzyme Assay.** The previously described (24) assay of aryl hydrocarbon hydroxylase activity was modified slightly. The reaction mixture, in a total volume of 1.00 ml, contained 50 μmoles of Tris chloride buffer, pH 7.5, 0.54 μmole of NADPH, 3 μmoles of MgCl₂, 0.10 ml of a 105,000 X g supernatant from a rat liver homogenate (containing approximately 18 mg protein/ml), 0.10 ml of placenta homogenate (containing 0.5 to 2.0 mg protein), and 80 μmoles of benzo[a]pyrene in 40 μl of methanol (added just prior to incubation). The mixture was incubated, with gentle shaking, at 37°C for 20 min, in air. The reaction was stopped by adding 1.0 ml of cold acetone. Enzyme activities were determined in duplicate and with several concentrations of placenta protein per flask. The activity was compared to a blank to which acetone had been added prior to incubation, as well as to a blank incubated with a complete reaction mixture minus the placental preparation. The remainder of the assay was carried out as previously described (24).

Hemoglobin concentrations were determined by a modification (33) of Drabkin’s technic (9). Crystalline bovine serum albumin was used as the reference standard for the modified Lowry protein determination. One unit of aryl hydrocarbon hydroxylase activity is defined as the amount of enzyme catalyzing the formation, during the 20-min incubation at 37°C, of a hydroxylated product with a fluorescence equivalent to 1 μmole of 3-hydroxybenzo[a]pyrene. Studies of the enzyme properties, enzyme stability, and enzyme inhibition were performed with placentas relatively free of hemorrhagic areas.

**RESULTS**

Chart 1 demonstrates the linearity of enzymatic hydroxylation with respect to the time of incubation. Product formation was linear for at least 20 min. The presence of rat liver 105,000 X g supernatant in addition to NADPH produced a 15 to 45% increase in placenta aryl hydrocarbon hydroxylase activity; the placental hydroxylase was always assayed in the presence of this fraction. No activity was observed in the presence of the rat liver supernatant when the placental homogenate was omitted.

Chart 2 illustrates the relationship between enzymatic activity and the amount of placenta protein present in the reaction mixture. A linear relationship was observed between the amount of product formed and the amount of placental enzyme present. This relationship was observed with placental preparations of either high or low hydroxylase activity. Also, within the limits of the assay system for aryl hydrocarbon
hydroxylase activity, the linear relationship was observed in placentas obtained from either smokers (lines A-D) or nonsmokers (line E). A measurement of less than 4 units of hydroxylase activity was regarded as insignificant enzymatic activity.

The stability of the placental aryl hydrocarbon hydroxylase was measured at various temperatures. The rates of loss of enzymatic activity were studied in placental preparations from smokers only, with the enzyme activities of the fresh material ranging from 42 to 166 units/mg protein. The relative half-life of placental aryl hydrocarbon hydroxylase activity at 0, -20, and -70°C was approximately 2, 12, and 24 days respectively (Chart 3). Thus, storage of the enzyme at -20°C for 12 days or at 0°C for 2 days results in a 50% decrease in enzyme activity. This relative instability of the enzyme system suggests that the handling of the tissue preparation may be a considerable factor in the observed variations in enzyme activity.

### Aryl Hydrocarbon Hydroxylase Activity in Placenta

Table 1 summarizes the aryl hydrocarbon hydroxylase activity measured in all 97 placentas. A positive relationship was observed between a relatively high level of placental aryl hydrocarbon hydroxylase activity and a history of cigarette smoking. The mean aryl hydrocarbon hydroxylase activity in the placentas from smoking women was 2- to 25-fold greater than that in the placentas from nonsmokers. The placentas from women who smoked 20 or more cigarettes per day showed a mean hydroxylase activity 5- to 25-fold greater than that found in placentas from women who did not smoke. Thus, our results generally confirm those of Welch et al. (42, 43). However, our results differ in that significant aryl hydrocarbon hydroxylase activity was found in the placentas of some women who smoked 20 or 30 cigarettes per day. This is contrary to the reports of Welch et al. (42, 43), who found no activity in the placentas from nonsmokers and activity in all of the placentas from smokers.

<table>
<thead>
<tr>
<th>Estimated number of cigarettes smoked daily</th>
<th>Number of placentas</th>
<th>Aryl hydrocarbon hydroxylase specific activity (units/mg protein)</th>
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<tr>
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<td>X</td>
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<td>40</td>
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Summary of aryl hydrocarbon hydroxylase activities assayed from all placentas collected.

*Without exception, the patient stated that she smoked approximately the same number of cigarettes during the last day of gestation, as compared to the daily total during the final week of gestation and to the number smoked during the entire gestational period (according to clinical histories taken).

A unit of human placenta aryl hydrocarbon hydroxylase activity has been arbitrarily defined as that amount catalyzing the formation during the 20-min incubation at 37°C, of hydroxylated product causing fluorescence equivalent to that of 1 μmole of 3-hydroxybenzo[a]pyrene.

The aryl hydrocarbon hydroxylase activities listed in Table 1 represent the activity of a homogenized segment of placenta assayed at two or more levels of protein. In one experiment, three or more separate segments from each of five placentas from smokers were assayed on the same day. The observed hydroxylase activity in different segments of the same placenta varied by as much as 35%. Also, we found that aryl hydrocarbon hydroxylase activity measured in a homogenate of a 35-gram segment of placenta was usually higher (30 to 80%) than the activity found in a homogenate from a 5-gram segment of the same placenta. These observations suggest the possibility of either a heterogeneous distribution of aryl hydrocarbon hydroxylase or factors affecting its activity in the placenta. Placental tissue consists of amnionic and chorionic membranes and cotyledons composed of chorionic villi; the villi include a large network of blood capillaries, with varying degrees of mesenchymal cells, Hofbauer cells, syntrophoblasts, and fibrinoid (30). We do not know the intercellular or intracellular distribution of the aryl hydrocarbon hydroxylase.
of placental tissue. Smooth endoplasmic reticulum in the
trophoblastic cell has been described (37, 44). However,
placenta aryl hydrocarbon hydroxylase activity might not be
localized entirely in the microsomal fraction, as is the case in
the liver. For example, in the adrenal cortex cell, hydroxyl-
ation requiring P-450 is localized in the outer mitochondrial
membrane (26, 29).

Charts 4 and 5 show the distribution of aryl hydrocarbon
hydroxylase in placentas assayed within 24 hours after
delivery and those assayed between 2 and 7 days after
delivery. As suggested by the stability studies, the hydrox-
ylase activity of placentas assayed at later times (Chart 5) is
lower than those assayed within 24 hours (Chart 4). The
correlation coefficient r where zero implies no prediction, and
unity, perfect prediction, was 0.53 for the distribution shown
in Chart 4 and 0.27 for the aryl hydrocarbon hydroxylase
distribution of Chart 5; these correlation coefficients represent
probabilities of P < 0.001 for Chart 4 and P = 0.10 for Chart
5. Chart 4 shows that of 24 placentas from nonsmokers, 18
showed essentially no detectable hydroxylase activity, i.e., a
specific activity of less than 5 units/mg protein. Of 22
placentas from women who smoked 20 or more cigarettes per
day, only 3 showed aryl hydrocarbon hydroxylase activity of
less than 5, and 16 showed hydroxylase activity ranging from
16 to 170 units/mg protein. The highest specific activity
observed in a nonsmoker was 32 in one case, and in 5 other
placentas from nonsmokers hydroxylase activity ranged from
10 to 28 units/mg protein. The data in Chart 5 show that the
placental tissue assayed 2 to 7 days after delivery generally
exhibited a much lower hydroxylase activity than the placen-
tal tissue assayed within 24 hours after delivery (Chart 4).
Also, the differences observed between the activity of placen-
tas from smoking and nonsmoking women was not as great
when assayed at 2 to 7 days as that observed when the assay
was performed within 24 hours after delivery. Thus, our
results show a substantial correlation between placental aryl
hydrocarbon hydroxylase activity and a history of cigarette
smoking.

A number of the patients were given various drugs during the
course of the pregnancy. These included phenobarbital,
chlorpheniramine, meprobamate, prophyprphene HCl,
promazine HCl, propylthiouracil, diphenhydramine, hydro-
chlorothiazide, and ampicillin. Also, during labor all of the
patients received either meperidine HCl and/or promethazine
HCl followed by regional anesthesia. We did not have large
efficient numbers of patients on each drug to assess the
potential effects of various drugs on placental aryl hydro-
carbon hydroxylase. However, we did not observe any effect
which appeared to be due to the use of a specific drug. For
example, two nonsmoking women to whom phenobarbital was
administered throughout the pregnancy did not show elevated
placental aryl hydrocarbon hydroyxylase. Phenobarbital
administration to women (21) during the final month of
pregnancy lowered the serum bilirubin levels of infants during
the first 4 days of life; this study suggests that phenobarbital
may have a transplacental effect of enhancing biliary excretion
and/or hepatic metabolism of bilirubin in the fetus (21).
Phenobarbital is an inducer of aryl hydrocarbon hydroxylase
activity (4), but at the usual clinical doses used, the drug did
not appear to stimulate placental hydroxylase. These findings
are consistent with the findings on one patient in the Welch et
al. study (46) and with our observations that chronic
administration of phenobarbital to pregnant hamsters does not
stimulate placental aryl hydrocarbon hydroxylase activity.
However, phenobarbital treatment of pregnant hamsters does
cause significant aryl hydrocarbon hydroxylase induction in maternal liver and lung as well as in fetal liver. 6

The age of the patients in this study ranged from 17 to 42 years, with a mean of 24.6 years. The race distribution of the patients was 89 Caucasians, 7 Negroes, and 1 Oriental. We did not have a sufficient number of patients to adequately assess the possible factors of race, age, or nutritional or medicinal status of the patient. No major differences were observed in patients of different race or age.

DISCUSSION

The data presented demonstrate a statistically significant relationship between a high level of aryl hydrocarbon hydroxylase activity in human placenta and a history of cigarette smoking in the mother. We observed no activity in the placentas obtained from some of the women with a history of cigarette smoking, and the placentas of some nonsmokers showed significantly measurable aryl hydrocarbon hydroxylase activity. Some of the minor differences between our results and those of Welch et al. (42, 43) may be due to some variations in the method of tissue handling and in the hydroxylase assay procedure. Thus, the time between delivery and the enzyme assay, the storage conditions of the tissue, the amount of blood present in the placenta homogenate, and variations in the assay might all be technical factors which influence the measurement of hydroxylase activity. The tar content of the cigarettes smoked, the depth of inhaling by each patient, genetic variations between individuals, and the patient's nutritional, medicinal, and dietary status may be additional factors which affect the placental induction of this enzyme. Furthermore, we know little about possible potentiating or antagonistic effects of other drugs or of elements present in cigarette smoke other than polycyclic hydrocarbons. There is a clear genetic basis for different responses to drugs (19). A study of identical and fraternal twins has demonstrated a genetic basis for the differences in the rate of phenylbutazone metabolism (36). Nutritional and dietary factors also affect the microsomal drug-metabolizing enzymes (4). Rats starved for 72 hours or fed a fat-free diet show a 15-fold decrease in aryl hydrocarbon hydroxylase activity in duodenal mucosa (40). Flavones and other natural food products have been found to stimulate the activity of this system in rats (41). While liver microsomes are stimulated by ethanol and metabolize ethanol in a system distinctly different from alcohol dehydrogenase in the rat and in man (20), the induction of hepatic aryl hydrocarbon hydroxylase by the feeding of ethanol was observed in rats but not in human volunteers (32). Although our data do not signify which agents in cigarette smoke may be responsible for the increased aryl hydrocarbon hydroxylase in placenta, it is reasonable to postulate that polycyclic hydrocarbons are the active inducers since they are present in cigarette smoke and are known inducers of aryl hydrocarbon hydroxylase in a variety of tissues in many species (4, 10).

The relationship of the hydroxylase enzyme to carcinogenesis requires much clarification. The enzyme is quite ubiquitous. It has been shown to be present in a variety of tissues by histochemical technics (38), and we have found the enzyme present in numerous tissues of at least four different species (D. W. Nebert and H. V. Gelboin, unpublished data). 6

The enzyme is detectable in most of the tissues examined. Furthermore, the hydroxylase activity is highly inducible, and the ability to induce the enzyme varies in different species and in different tissues. 5 The microsomal enzyme derived from rat liver also catalyzes the formation of hydrocarbon DNA complexes (11). Aryl hydrocarbon hydroxylase may thus be important in the binding of hydrocarbon to DNA in vivo. The enzyme is also present and inducible in a variety of nonhuman cell types grown in culture (25). The cells that contain the enzyme are generally sensitive to the toxic action of the polycyclic hydrocarbons. The aryl hydrocarbon hydroxylase, however, is lacking in at least five cell lines grown in culture (25), and these cells are resistant to the toxic action of the hydrocarbons. Thus, there appears to be a positive relationship between the toxic action of the polycyclic hydrocarbons and the presence of the enzyme system. This relationship may be also true for the carcinogenic activity of the polycyclic hydrocarbons. With other experimental conditions, the induction of the microsomal oxidative enzyme system by polycyclic hydrocarbons seems clearly responsible for the inhibition of carcinogenesis by these agents of either aminoazo dyes (10, 23) or 2-acetylaminofluorene in rat liver (7, 10, 23). Hence, the nature and level of the enzyme activity in a given tissue may be uniquely relevant to the susceptibility of that tissue to chemical carcinogenesis. Thus, more information about the profile of metabolic products of the control and induced enzyme in each tissue and species, the nature of the carcinogenic intermediate, and, if possible, the characteristics of the enzyme with respect to the formation of each product is necessary. Furthermore, it may be possible to develop conditions to increase the ratio of nontoxic to toxic, or carcinogenic, metabolites or to selectively inhibit the aryl hydrocarbon hydroxylase system. In this manner the carcinogenic activity of the polycyclic hydrocarbons may be diminished.

Placental enzymes and especially those metabolizing foreign compounds also may be significant factors in the resistance of the developing fetus to potentially toxic or teratogenic substances. The teratogen (18) may be inaccessible to the fetus because of its enzymatic conversion to nontoxic, more polar derivatives by the placental enzyme system. On the other hand, the placenta conceivably could metabolize an inactive compound to an active teratogen. In the case of teratogens affecting human embryogenesis, the placental enzymes present and their ability to be induced during the first nine weeks of gestation might be more relevant. The different types of cells in the placenta may have markedly different metabolic potentials (37), and the overall functions of the placenta probably undergo extensive changes over the course of gestation.

The metabolism of endogenous substrates (15, 22, 34) and xenobiotic substances (17, 35, 45) by human placenta has only recently been studied. Although the carbon monoxide-binding pigment P-450 (4, 10, 13, 27-29) was not detected in human placentas of 9 to 12 weeks' gestation (17), it was found in subcellular fractions of human placenta at term (22). The present study and that of Welch and coworkers (42, 43)
demonstrate that an oxidative pathway exists in human placenta at term. The oxidative enzyme pathways in human placenta may be important in maintaining the proper hormonal balance required for the normal growth and development of the fetus. The hydroxylation of polycyclic hydrocarbons and steroid hormones may share common enzyme systems (4, 8, 10, 13). The association of maternal smoking with decreased weight of the newborn has been reviewed by Comstock and Lundin (3). The average birth weight decrease in the newborns of women smoking cigarettes has been reported as 229 grams by Zabriskie (46), and 129 grams by Abernathy et al. (1), who used multiple regression analysis. A recent report from the Collaborative Study on Cerebral Palsy (2), where multiple regression analysis was also used, predicts a birth weight decrease of 8 to 9 grams per cigarette smoked daily. Thus, a woman smoking 20 cigarettes a day can expect to have a baby lighter in weight by 165 to 180 grams.

Ravenholt et al. (31) concluded that maternal smoking was associated with an increased fetal death rate, while Comstock and Lundin (3) felt that any increase in fetal deaths among newborns of women who smoke was related to some other factor such as adequacy of prenatal care or postnatal environment and care. The incidence of prematurity among newborns of women who smoked during pregnancy was 2.5 times greater than that among newborns of nonsmokers (46). This observation might be due in part to the statistical decrease in fetal weight. Delivery before term also might reflect a critical change in endocrine balance between the mother and fetus, such that labor and delivery occurs earlier. We do not know the possible relationship between birth weight, prematurity, and fetal death rate and the level of placental aryl hydrocarbon hydroxylase activity. This merits further study.

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