Determinants of Polycyclic Aromatic Hydrocarbon-DNA Adducts in Human Placenta

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ABSTRACT

To determine the relative contributions of tobacco smoking and P-450 metabolism (cytochrome P-450(1A1)) in the formation of benzo(a)pyrene-diol-epoxide and other polycyclic aromatic hydrocarbon-DNA adducts in vivo, 16 human placentas were assayed for aryl hydrocarbon hydroxylase activity and (±)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene-DNA adduct levels. Immunoaffinity chromatography columns, conjugated with monoclonal antibodies raised against benzo(a)pyrene-diol-epoxide-deoxyguanosine, were used to concentrate polycyclic aromatic hydrocarbon-DNA adducted nucleotides, and synchronous fluorescence spectroscopy was used specifically to detect 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BP-tetrol) extracted from acid hydrolysates of immunocentrifuged materials. Data were analyzed for associations with maternal dietary and smoking habits, umbilical cord blood cotinine levels, and placental aryl hydrocarbon hydroxylase levels. Complex mixtures of fluorescent materials were present in organic solvent extracts of acid hydrolysates of immunocentrifuged nucleotide-adducts from all placentas with patterns of fluorescence that may be associated with tobacco smoking determined by generation of spectral fluorescence excitation-emission matrices. BP-tetrols were detected in extracts from 8 placentas: 5 of 7 from smokers and 3 of 9 from nonsmokers. Placental aryl hydrocarbon hydroxylase activity was significantly higher in placentas from which BP-tetrols were extracted (1.30 ± 0.40 (mean ± SE) pmol 3-hydroxybenzo(a)pyrene mg protein−1 min−1), than among placentas from which BP-tetrols were not extracted (1.37 ± 3.73 pmol 3-hydroxybenzo(a)pyrene mg protein−1 min−1) (P = 0.03, Student’s t test). This association was independent of maternal smoking or umbilical cord blood cotinine levels. These results indicate that while maternal tobacco smoking is associated with the accumulation of putative, but as yet unidentified, polycyclic aromatic hydrocarbon-DNA adducts in placenta, metabolic capacity appears to be the principal determinant for the (±)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene-DNA adduct levels detected.

INTRODUCTION

Tobacco smoke and other complex mixtures associated with human cancers contain BP and other PAHs. Genotoxicity of PAHs depends on metabolism to diol-epoxides that bind covalently to DNA (1). To understand risks associated with PAH exposures, several methods have been developed to detect adducts formed by these agents in the DNA of target or surrogate tissues (2–4). These assays have provided evidence for putative PAH-DNA adducts in human lymphocytes (5, 6), lung (7–9), and placenta (10). We have previously used IAC and HPLC linked with SFS to extract and detect BP-tetrol, a derivative of BPDE-DNA, from human lung and placenta. Together with corroborative gas chromatography/mass spectroscopy data, these former studies positively identified BP exposure as a potential risk to humans (11, 12).

In experimental systems, both PAH-DNA adduct levels and toxicity are determined by multiple factors, including level and route of exposure, age, and metabolic phenotype. Furthermore, metabolic phenotypes are themselves multifactorial. In the case of PAH-genotoxicity, the determining phenotype reflects genetically regulated induction of cytochromes P-450 that bioactivate xenobiotics. These are predominantly isoenzymes from the cytochrome P-450 family that can respond to agents that induce or inhibit their activity (1, 13). In humans, inducibility of CYP1A1 has been positively correlated with risk for cancer among tobacco smokers (14, 15), but a relationship between induction of this enzyme system and in vivo adduct formation has not been clearly demonstrated. Furthermore, these studies may yield important information that bases upon transplacental carcinogenesis.

Human placenta is a readily available, CYP1A1-inducible tissue that can serve both as a dosimeter for epidemiological studies (10, 12) and as a model in which environmental and genetic determinants of carcinogen toxicity can be investigated (16). In addition, the presence of inducible enzymes at the interface between maternal and fetal circulations that bioactivate xenobiotics may be an important determinant of transplacental DNA damage and carcinogenesis by PAHs (17). The purpose of this investigation was to assess the relative contributions of several factors—including maternal tobacco use, placental metabolic phenotype, and diet—to the formation of BPDE-DNA adducts in placenta.

MATERIALS AND METHODS

Chemicals and Reagents. Nucleic acid grade phenol was obtained from Bethesda Research Laboratories (Gaithersburg, MD). Solvents (chloroform, isooamyl alcohol, and methanol) were purchased from Baker Scientific (Phillipsburg, NJ). Racemic BPDE, its tritium labeled analogue ([3H]BPDE), and 3-OH-BP were obtained through the Chemical Carcinogen Standard Repository at the National Cancer Institute (Bethesda, MD). Calf thymus DNA, NADH, and NADPH were purchased from Sigma Chemical Company (St. Louis, MO). Calf thymus DNA was treated with BPDE or [3H]BPDE according to published methods to provide highly modified standard DNA (0.4–1.0%) (18, 19). Enzymes (P1-nuclease, calf spleen phosphodiesterase, RNase A, proteinase K, and micrococcal nuclease) were obtained from Calbiochem (La Jolla, CA). Antibody isolation columns were purchased from Isolab, Inc. (Akron, OH) and cyanogen bromide activated Sepharose 4B was purchased from Pharmacia, Inc. (Piscataway, NJ).

Placental Tissue, DNA, and Microsomes. Human placentas were collected at birth by experienced research nurse specialists from term, uncomplicated deliveries at University Hospital, Denver, CO. Blood
and tissue samples were obtained with informed consent according to a protocol approved by the Human Subjects Committee, University of Colorado Health Sciences Center. Umbilical cord blood (~20 ml) was collected into heparinized containers through venipuncture of the umbilical vein at its insertion into the fetal surface of the placenta. Each placenta was then turned over and 5 normal villous tissue samples (~20 g each) were cut from 1 central and 4 equally spaced peripheral sites. Each tissue sample was then divided and the halves pooled. One pool was immediately placed in ice-cold saline (0.9%) for preparation of syncytiotrophoblast nuclei, and the other half frozen (~80°C) for later extraction of microsomes. Banded nuclei were prepared by differential centrifugation (21) after thawing and homogenizing villous samples at a ratio of 1:4 (w/v) in sodium phosphate-KCl (50 mM; 1.15%, pH 7.4).

Maternal Histories. Each mother participating in the investigation was interviewed postpartum by the nurse specialists who administered a questionnaire. Information recorded included maternal age, race, residence, occupation, and pregnancy and medical history. Histories also included descriptions of tobacco use during the pregnancy, diet (general description and sources, and intake of green vegetables and consumption of charbroiled meats during the pregnancy), use of marijuana, and use of prescription and over-the-counter medications. For this investigation, charbroiled meat intakes were classified as "regular" (general description and sources, and intake of green vegetables and consumption of charbroiled meats during the pregnancy), or "occasional" if they were consumed once within 2 weeks to 2 months. Avoidance was also documented.

Immunoadfinity Chromatography and HPLC. Samples of placental DNA (1 mg) were digested with micrococcal nuclease, calf spleen phosphodiesterase, and P1-nuclease under conditions described previously (22). Digests were applied to immunoadfinity columns bearing monoclonal antibodies (8E11) raised against BPDE-deoxyguanosine (23). The columns were washed extensively with Tris buffer (10 mM; pH 7.4), and bound materials were eluted with NaOH (50 mM). Fractions that eluted in base were acidified with HCl (final concentration, 100 mM) and heated (90°C, 3 h). The acid hydrolysates were generated in the synchronous mode using a Perkin-Elmer fluorescence spectrophotometer (MFP-66; Perkin Elmer Corp., Rockville, MD).

Prior to HPLC, fluorescence excitation-emission matrices were generated for acid hydrolyzed, organic extracts of IAC-concentrated nucleotide-adducts. An initial synchronous fluorescence spectrum was generated using a wavelength difference of 10 nm, and then 29 successive synchronous fluorescence spectra were generated for the same sample by incrementing the wavelength difference by 4 nm for each spectrum. contour plots of these fluorescence data were prepared using an Apollo workstation (Star Technologies, Sterling, VA). Measurement of BP-tetrol was made in HPLC fractions by recording fluorescence spectra generated by driving the excitation and emission monochrometers at a constant wavelength difference (6A) of 34 nm. Under these conditions, the pyrene signature signal of extracted BP-tetrol was maximal at emission wavelength 379 nm (excitation wavelength, 345 nm) (4, 11, 12, 24). This signal is proportional to the concentration of BP-tetrol that is quantitated from a standard curve generated with authentic BP-tetrol (22, 25).

Placental Aryl Hydrocarbon Hydroxylase. Placental AHH activity was measured in pmol 3-hydroxybenzo(a)pyrene generated per min per mg microsomal protein, according to the technique of Vaugh et al. (26) as described previously (27). Microsomal protein was determined using commercial reagents (Bio-Rad Laboratories, Richmond, CA).

Plasma Cotinine Concentration. Umbilical cord blood cotinine concentrations were measured at the American Health Foundation using a radioimmunoassay. The limit of detection for this assay is 2 ng/ml (28).

Data Analyses. A stepwise regression model was used to evaluate contributions of multiple factors to placental BPDE-DNA adduct levels (29). Subset analyses were performed using Fisher's exact test and Student's t test procedures. Data were log transformed where appropriate to obtain homogeneity of variance.

RESULTS

Benzo(a)pyrene-Diol-Epoxide-DNA Levels. A total of 106 placentas were collected according to the protocol described above. Levels of BPDE-DNA adducts were determined by SFS following concentration of PAH-DNA adducts by IAC, hydroslysis to tetroks (0.1 N HCl), and purification of BP-tetrols by HPLC (22). Table 1 records BPDE-DNA levels, cord blood cotinine levels, and placental AHH activities for 16 samples selected randomly and without knowledge of maternal histories. Measurable quantities of BP-tetrol were extracted from 1 mg DNA in 8 placentas.

Adducts were detected in placental DNA from more smokers (5 of 7) than nonsmokers (3 of 9), but these frequencies did not reach significance. When AHH activities in placentas in which

<table>
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<th>Case</th>
<th>Maternal tobacco use (cigarettes/day)</th>
<th>BPDE-DNA a (fmol/mg DNA)</th>
<th>AHH (pmol 3-OH-BP mg -1 protein min -1 )</th>
<th>Cotinine b (ng/ml)</th>
<th>Diet (charbroiled meat consumption)</th>
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a Rank order of study for smokers, then nonsmokers.
b Limit of detection for radioimmunoassay is 2 ng cotinine/ml serum.
c ND, not detected (below limit of assay sensitivity).

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BPDE-DNA adducts were detected compared to placentas in which BPDE-DNA adducts were not detected (Table 1), the mean level [13.0 ± 4.0 (mean ± SE) pmol 3-OH-BP mg protein"1 min"1] among positive placentas was significantly greater than the mean level (1.3 ± 3.7 pmol 3-OH-BP mg protein"1 min"1) among negative placentas (P = 0.03, Student's t test). Multiple regression analysis in which variables were added stepwise to evaluate contributions of maternal exposures to variance in BPDE-DNA levels failed to identify any single significant determinant of BPDE-DNA adduct levels, but AHH activity was identified as the strongest contributing factor. In addition, a significant association between AHH activity and cotinine level was identified (P < 0.05).

Fluorescence Excitation-Emission Matrices. Eluates from IAC columns contained adducted nucleotides which, following acid hydrolysis and organic solvent extraction, yielded mixtures of unidentified fluorescent materials. Prior to HPLC separation, fluorescence excitation-emission matrices were found to be complex (Fig. 1). Consequently, signature signals for BP-tetrols at a λ of 34 nm were not always discernable in these complex mixture samples prior to HPLC. Specific signature signals other than those characterizing BP-tetrols were not identified, however, some patterns of fluorescence were common to different samples. Fig. 1 compares the fluorescence excitation-emission matrices for extracts of human cells cultured in vitro (Fig. 1a), BPDE modified DNA (Fig. 1b), and 3 placentas from which BP-tetrol was ultimately extracted (Fig. 1, c-e) and measured following HPLC. Fig. 1c depicts the fluorescence spectrum from a placenta (Table 1, case 10) that was obtained from a nonsmoking woman who ate a vegetarian diet. A prominent BP-tetrol signature signal is evident (excitation, 345 nm; emission, 379 nm with λ34 nm), compared to Fig. 1a, in which no BP-tetrol signature is present (Fig. 1, boxes marked A). In Fig. 1, d and e, similar fluorescence patterns from placentas 3 and 6 (Table 1) obtained from smoking women are seen. In these samples, signals with excitation peaks between 300 and 425 nm were similar at λs between 55 and 125 nm (Fig. 1 boxes marked B). These signals may indicate the presence in human placenta of PAH-DNA adducts other than BPDE-DNA.

DISCUSSION

The present study investigates the contributions of multiple factors in the accumulation of BPDE-DNA adducts in human placenta. Synchronous fluorescence spectroscopy when combined with HPLC (and in this case IAC) is a highly specific technique for the measurement of BPDE-DNA adducts. The results of these studies indicate that active tobacco smoking is not the sole source of BPDE-DNA adducts in humans (Fig. 1). The data also demonstrate the formation of PAH-DNA adducts in addition to those of BP in humans. Studies including measures of AHH activity clearly indicate that metabolic phenotype is an important determinant of BPDE-DNA adduct levels.

Eight of 16 placentas examined in this study yielded BP-tetrol derived from BPDE-DNA. Adducts of BPDE-DNA were detected in 5 of 7 placentas from cigarette smokers and 3 of 9 placentas from nonsmokers. Thus, activities other than current tobacco use expose some women to levels of BP high enough to produce comparable genotoxic damage. These findings are consistent with the widespread distribution of BP in the environment, including food items (30). They extend previous reports in which BP-tetrol was extracted from DNA pooled from placentas of both smokers and nonsmokers (11, 12), and show a similar trend in that cigarette smoking per se is not closely correlated with BPDE-DNA adduct formation (31).

The present application of IAC and SFS to analysis of human placental DNA measured BPDE-DNA adducts at levels approximately 10-fold lower than the placental PAH-DNA adducts reported by enzyme-linked immunosorbent assay (10) and antibody-based fluorimmunosensor studies (32) using polyclonal antibodies raised against BPDE-DNA. These differences are likely to be due to cross-reactivity of anti-BPDE-DNA antibodies with other PAH-DNA adducts (11, 33-35). Adducted nucleotides were concentrated by these antibodies from all placentas, and extracts of these contained complex mixtures of fluorescent compounds. Fluorescence spectral analyses of these mixtures are interesting and may eventually assist in the identification of specific xenobiotics or their source. In Fig. 1 (c-e), all 3 contour plots are from extracts of IAC concentrated materials that contained BP-tetrol. The fluorescence patterns of the samples from smokers (Fig. 1, d and e) are similar, especially at the larger constant wavelength differences (Fig. 1, boxes marked B). Similarities are also noted with previously published data in placenta from smokers (27). Repeated occurrence of these patterns for extracts from smokers in fluorescence spectral excitation-emission matrices suggests that this type of analysis may be informative concerning the source of exposure giving rise to adducts in vivo. The patterns seen may be analogous to patterns of adducts detected by the 3P-postlabeling assay in placental DNA (10, 36).

Benzo(a)pyrene metabolites are known mutagens and carcin-
HYDROCARBON-DNA ADDUCTS IN HUMAN PLACENTA


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