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Human Placental Microsomal Activation and DNA Adduction by Air Pollutants

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Assessing human risks from exposures to complex emissions is complicated by the fact that a myriad of chemicals including potent procarcinogens such as polycyclic aromatic hydrocarbons (PAHs) are present in these mixtures. Testing isolated chemical constituents is not always appropriate, since chemical interactions occurring within the mixture can influence the genotoxic properties of the chemical. Benzo[*a*]pyrene (B[*a*]P), a common combustion emission constituent, and a widely studied carcinogenic PAH is often considered as an index of environmental pollution. Evidence suggests measurement of B[*a*]P exposure alone does not always correlate with the total PAH exposure (Peterson et al. 1988). This may be because B[*a*]P has been estimated to constitute only 10-13 % of the total PAHs concentrations in the airborne total particulate matter (Binkova et al. 1994). To evaluate human risks to the genotoxic effects of exposure to complex mixtures, studies have been conducted on the comparative assessment of mixtures in short-term genetic bioassays and rodent models (Lewtas et al. 1993).

Covalent formation of DNA adducts by xenobiotics represents a critical step in the initiation of carcinogenesis (Harris et al. 1991). In human placental DNA, various adducts of unknown chemical structure have been detected by the ^{32}P-postlabeling method (Everson et al. 1986; Manchester et al. 1990) and some of these adducts have been found to be increased in placentae of smoking women (Everson et al. 1986). DNA adducts have also been detected in the umblical cord blood vessels (Hansen et al. 1992), and in the lung and liver of aborted fetus (Hatch et al. 1990) from smokers. It has been shown that the human placenta possesses the ability to metabolize numerous xenobiotics and endogenous steriods to DNA reactive species (McRobie et al. 1996; Pasanen and Pelkonen 1994). Furthermore, maternal cigarette smoking increases the expression of cytochrome P450 (CYP) genes including CYP1A1 in human full-term placenta (Boden et al. 1995). The human CYP1A1 gene codes for an inducible enzyme system involved in the biotransformation of certain xenobiotics, including PAHs; some of the metabolites are carcinogenic and/or mutagenic (Whyatt et al. 1995).

The ^{32}P-postlabeling assay is highly sensitive and can detect DNA damage resulting from PAHs at levels approximating one adduct in 10^9 unmodified nucleotides

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(Randerath et al. 1985; Gupta 1985), and has been used to measure the relative DNA reactivity of metabolized complex chemical constituents of environmental mixtures *in vivo* and *in vitro* (Lewtas et al. 1993) and in efficacy studies to screen for potential cancer chemopreventive agents *in vitro* (Smith and Gupta 1996). Placental metabolism of xenobiotics is of importance since it has been shown that drugs taken during pregnancy are metabolized in the placenta and can cross over into the fetal circulation (Juchau 1978). Many such transplacental environmental chemicals can be teratogenic and / or carcinogenic to the developing human fetus. In this investigation, we studied whether placental microsomes pooled from smokers have sufficient metabolic activation to induce DNA adduct formation as measured by ^{32}P-postlabeling in DNA modified by environmental carcinogen B[*a*]P and three complex mixture particle extracts; one point source: coke oven and two mobile source: diesel particle extract # 1 and # 2. The results demonstrate that placental microsomes of actively smoking mothers have sufficient activity for metabolic activation of the PAH constituents within the mixtures and as such can be used to 1) compare DNA reactivity of other environmentally relevant complex mixtures, 2) compare microsomal activation derived from other target organs, 3) generate quantities of modified DNA required to characterize specific adducts.

MATERIALS AND METHODS

All chemicals and reagents were of analytical or enzyme grade unless otherwise specified. Polyethyleneimine (PEI)-cellulose thin layer chromatography (TLC) plates were prepared essentially as described previously (Gupta et al. 1982). Micrococcal nuclease (MN), [γ^{32}P]ATP (sp. act. >3000 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Calf thymus DNA, tris(hydroxymethyl) aminomethane, Na_2EDTA, lithium chloride, zinc chloride, calcium chloride, magnesium chloride, mono- and dibasic-sodium phosphate, sodium acetate, ammonium acetate, ethyl alcohol, urea, DL-isocitric acid and nicotinamide adenine dmucleotide phosphate (NADP) were purchased from Sigma Chemical Co. (St. Louis, MO). Calf spleen phosphodiesterase (SPDE) was obtained from Boehringer Mannheim (Indianapolis, IN) and T4 polynucleotide kinase from Pharmacia-PL Biochemicals (Piscataway, NJ). Glacial acetic acid, hydrochloric acid, purified 90% formic acid, ammonium hydroxide, lithium hydroxide, dichloromethane and 1-butanol were obtained from Fisher Scientific (Pittsburgh, PA). Isoamyl alcohol was procured from Mallinckrodt Inc. (Paris, KY) and redistilled phenol from Bethesda Research Laboratories (Gaithersburg, MD). Benzo[*a*]pyrene was purchased from the National Cancer Institute Carcinogen Standard Reference Repository (Bethesda, MD).

Diesel automotive emissions were obtained from light-duty passenger cars, Volkswagon turbo (diesel #1) and Nissan 220C (diesel #2). Emission particulates were collected on 20 x 20 Pallflex T60-A-20 teflon-coated glass-fiber filters at a flow rate of 100 cfm using the dilution tunnel sampling technique (Williams et al. 1986). The coke oven main emissions were collected from the separator between the gas collector main and the primary coolers within the coke oven battery located at a coke

oven in Gadsben, Alabama (Williams et al. 1986). The emissions were trapped in dichloromethane. After removal of the solvent by evaporation under nitrogen, the samples were stored at -80° C in the dark. All test sample extracts were dissolved in dimethylsulfoxide (DMSO) at a final concentration of 2 mg/ml and 20 µl aliquots from this stock solutions were used for *in vitro* human placental microsomal activation and modification of calf thymus DNA.

Human placentas from fill-term, uncomplicated pregnancies from active smokers were collected during parturation at the University Hospital, Denver, CO, according to a protocol approved by the University of Colorado Health Sciences Center Human Subjects Committee. Smoking histories included questions about tobacco use and active smoking was defined as daily use of cigarettes. Women who seldom smoked or quit smoking during pregnancy were excluded from the study. Placental microsomes were prepared immediately from villous samples by differential centrifugation as described previously (Manchester 1981). Samples were screened for aryl hydrocarbon hydroxylase (AHH) activity as previously described using B[*a*] P as substrate (Vaught et al. 1979). Microsomes with relatively high AHH activity (AHH activity 20 to 65 pmoles 3-hydroxy B[*a*]P formed per min per mg protein) were pooled for subsequent *in vitro* modification of calf thymus DNA by B[*a*]P and organic extracts of complex mixtures *viz.*, diesel and coke oven main as described previously (Vaught et al. 1979). Briefly, calf thymus DNA (1.2 mg) was reacted *in vitro* by incubation with either environmental samples (40 µg extract) or B[*a*]P (20 nmole) in the presence of placental microsomes (1 mg microsomal protein) in a total volume of 1 ml potassium phosphate buffer (0.8 M, pH= 7.4) containing 3.3 mM $MgCl_2$, 0.81 mM NADP, 17 mM DL-isocitrate, and 70 µg Sigma type 1V isocitrate dehydrogenase. The reaction was allowed to proceed for 45 min at 37°C. DNA was recovered from the incubation mixture by ethanol precipitation, rinsed with 70% ethanol, and then redissolved in 1.0 ml of TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH= 7.4).

DNA adducts were analyzed by ^{32}P-postlabeling assay using the 1-butanol extraction method (Gupta 1985) to isolate DNA adducts. Briefly, B[*a*]P or particle extracts-modified calf thymus DNA (5.0 µg) was hydrolyzed to 3'-mononucleotides by digestion with SPDE/MN DNA digestion was verified by labeling an aliquot of normal DNA nucleotides prior to adduct enrichment. Radiolabeled adduct nucleotide biphosphates were then separated by TLC on 10 x 10 cm PEI cellulose plates, using the following solvent system: D-1, 1 M sodium phosphate, pH=6.8, with overnight development onto a 10 cm Whatman grade 3MM Chr wick followed by a wash with water; D-2, predevelopment to 1 cm with 2.5 M ammonium formate, pH=3.5; D-3, 4 M lithium formate, 7 M urea, pH=3.5, followed by a wash with water; D-4, 0.8 M lithium chloride, 7 M urea, 0.5 M Tris-HCI, pH=8.0, followed by a wash with water; D-5, 0.4 M magnesium chloride, with development onto a 3 cm Whatman grade 3 MM Chr wick, followed by a final wash with water. DNA adducts were visualized by autoradiography carried out at -80°C. Areas of radioactivity associated with adducts on TLC plates were excised and then counted in a scintillation counter (Packard

1900CA, Downers Grove, IL) using 5 ml opti-fluor (Packard, II). Total relative adduct level (RAL) was calculated by dividing the amount of radioactivity in the adducted nucleotides by the total nucleotides after correction for the background activity and dilution factor (Gupta 1985).

RESULTS AND DISCUSSION

Benzo[*a*]pyrene and complex air pollution particles extract obtained from two sources (coke oven main and diesel) were metabolized by human placental microsomes to DNA reactive chemical species. Those putative electrophiles, which formed covalent adducts with DNA *in* vitro and were detected by the ^{32}P-postlabeling are shown in Figure 1 d-f. It is clear from the figure that there were several adducts formed that migrated as a diffuse diagonal zone of radioactivity (DRZ). The migration properties of DNA adducts that were formed when B[*a*]P was incubated with human placental microsomes and calf thymus DNA were similar to those of the adducts in the DRZ for coke oven extract-modified DNA (Fig. 1d). For B[*a*]P, the major adduct appears to be the result of binding of the bay-region diolepoxide of B[*a*]P to deoxyguanosine residues in DNA. When DNA alone was incubated with microsomes, two minor adducts (Fig. lb; spot #1 and 2) were detected, presumably arising from reactive intermediates that were sequestered in the microsomes. When B[*a*]P was incubated with calf thymus DNA in the absence of placental microsomes, neither DRZ nor discrete DNA adducts were detected (Fig. 1c). In diesel extract-modified DNA, a distinct, but relatively polar adduct was detected away from the adducts migrating within the DRZ (Fig. 1e-f; spot #1). This adduct was nuclease P1 sensitive in this study (data not shown). While the identification of this adduct in this study is indeed speculative and exact comparisons are not possible, the chromatographic properties of this adduct is very similar to the tentatively identified nitroarene derived-adducts detected in other studies using diesel particle extracts (Gallagher et al. 1991; King et al. 1993). Further studies to characterize the DNA adducts formed from these mixtures would provide additional information regarding the specific identity of the parent compounds from which the DNA reactive intermediates are formed.

Because the absolute levels of DNA adducts formed are a function of the relative concentration of the individual chemical constituents of the complex test mixtures, the ranking of these total adduct levels have been compared. Quantitatively, the total relative adduct level (RAL) as determined by the butanol enhancement of the ^{32}P-postlabeling method was found to be highest for the coke oven-modified DNA (30 ± 2.8 adducts/10^8 nucleotides) followed by diesel. Diesel #l modified DNA (17±1.5 adducts/10^8 nucleotides) showed approximately twice the level total DNA adducts, compared to diesel #2 (9±0.8 adducts/10^8 nucleotides). This difference in relative concentrations of DNA adducts in the two diesel samples may be attributed to relatively high concentrations of nitrated-PAHs present in diesel #1 compared to diesel #2 extracts (Williams et.al. 1986). Furthermore, B[*a*]P modified-calf thymus DNA in presence of placental microsomes, used as positive control, yielded 20±1.0

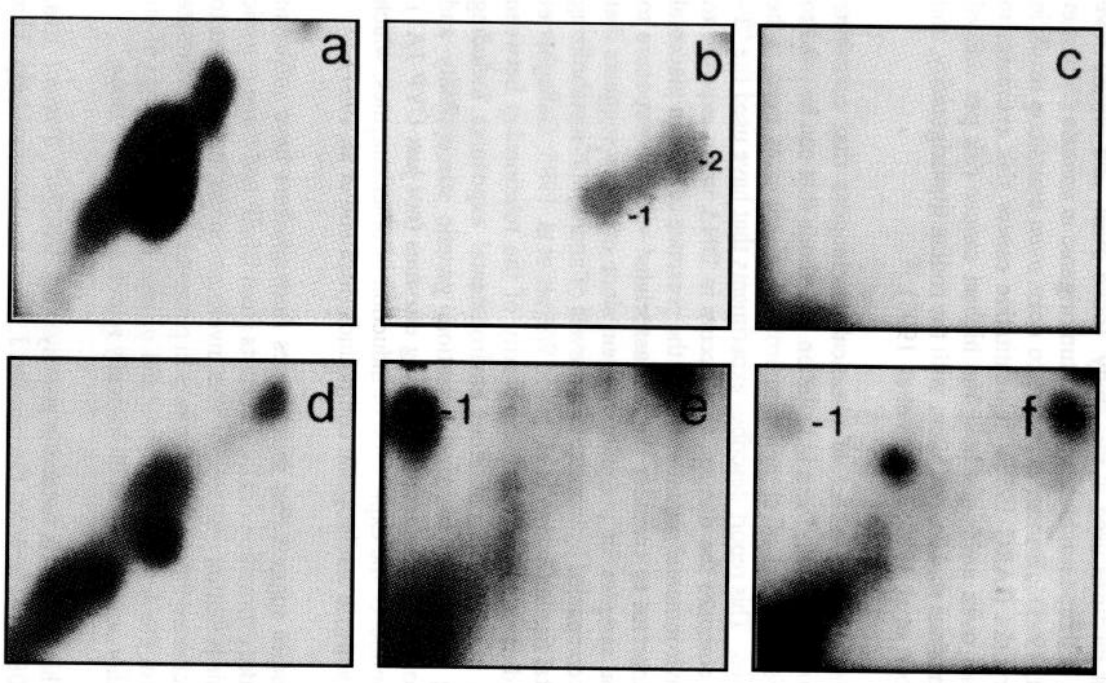

Figure1. ^{32}P-postlabeling autoradiograms of DNA adducts. Calf thymus DNA was treated with either air particle extracts in the presence of human placental microsomes (d-f), B[a]P (a), or calf thymus DNA treated with microsomes alone (b) B[a]P without human placental microsomes (c). The particulate extracts activated by human placental microsomes were: coke oven extract (d), diesel #1 particles extract (e), and diesel #2 particle extract (f). The direction of elution for solvent D-1 was top to bottom; D-3 was bottom to top; D-4 and D-5 was left to right.

adducts/10^8 nucleotides.

The complex pollutant mixtures used in this study (coke oven and diesel) have shown to be mutagenic in short-term bioassays and tumorigenic in animals (Albert et al. 1983; Lewtas et al. 1993). Human exposure to coke oven emissions result in increased lung cancer risks (IARC 1985). Quantitative cancer risk estimates in humans show that coke oven emissions have the highest cancer risk per unit of exposure among the emissions studied, consistent with the rodent tumorigenicity, and DNA adduct data (Albert et al. 1983; Lewtas et al. 1993).

It is difficult to assess the true effect of chemical carcinogens that constitute components of a mixture by testing them in isolation. Methods that can be used to evaluate the genotoxic potential of complex environmental mixtures need to be developed and established. This report describes experiments that have used the ^{32}P-postlabeling assay to compare the damage that occurs in DNA when exposed to complex mixtures of environmental contaminants in the presence of human placental microsomes. It is important to consider the risk associated with the exposure to complex environmental samples for pregnant women; since evidence suggests that maternal smoking is accompanied by increase in the levels of xenobiotic-metabolizing enzymes in human fetal tissue (Boden et al. 1995; Whyatt et al. 1995; Gallagher et al. 1994). These studies underscore the complexity of the relationship between enzyme induction as it relates to risk from environmental exposures including complications arising from diet, smoking cessation, genetic susceptibility and interactions between other carcinogen metabolizing enzymes (not just CYP 1A1), whose products may influence the expression or induction of other enzymes, which can either detoxify or activate carcinogenic compounds (McLeod et al. 1997).

In conclusion, our studies suggest that microsomes from smokers have sufficient metabolic activity to induce formation of DNA adducts from B[*a*]P and three organic extracts of complex air pollution mixtures. This investigation can be extended to study the extent to which inter-individual differences in placental AHH levels correlate with the ability to form DNA adducts *in vitro* and *in vivo* for a wide range of air pollutants using a readily obtainable and biologically relevant surrogate tissue.

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REFERENCES

Albert RE, Lewtas J, Nesnow S, Thorslund TW, Anderson E (1983) Comparative potency method for cancer risk assessment: application to diesel particulate emissions. Risk Anal 3:101-117

Binkova B, Dobias L, Wolff T, Sram RJ (1994) ^{32}P-postlabeling analysis of DNA adducts in tissues of rats exposed to coke-oven emissions. Mut Res 307:355-363

Boden AG, Bush PG, Burke MD, Abramovich DR Aggett P, Mayhew TM, Page KR (1995) Human placental cytochrome P450 and quinone reductase enzyme induction in relation to maternal smoking. Reprod Fertil Dev 7:1521-1524

Everson RB, Randerath E, Santella RM, Cefalo RC, Avitts TA, Randerath K (1986) Detection of smoking-related covalent DNA adducts in human placenta. Science 231:54-57

Gallagher JE, Kohan MJ, George MH, Lewtas J (1991) Improvement in the diagnostic potential of ^{32}P-postlabeling analysis demonstrated by the selective formation and comparative analysis of nitrated-PAH-derived adducts arising from diesel particle extracts. Carcinogenesis 12:1685-1691

Gallagher JE, Everson R, George M, Lucier G (1994) Comparison of DNA adduct levels in human placenta from polychlorinated biphenyl exposed women and smokers in which CYP 1A1 levels are similarly elevated. Teratog Carcinog Mutagen 14:183-192

Gupta RC, Dighe NR, Randerath K (1982) ^{32}P-postlabeling analysis of nonradioactive aromatic carcinogen-DNA adducts. Carcinogenesis 3:108-1092

Gupta RC (1985) Enhanced sensitivity of ^{32}P-postlabeling analysis for aromatic carcinogen: DNA adducts. Cancer Res 45:5656-5662

Hansen C, Sorensen LD, Asmussen I, Autrup H (1992) Transplacental exposure to tobacco smoke in human-adduct formation in placenta umblical cord blood vessels. Teratog, Carcinog, Mutagen 12:51-60

Harris CC (1991) Chemical and physical carcinogenesis: advances and perspectives for the 1990s. Cancer Res 51:5023-5044

Hatch MC, Warburton D, Santella RM (1990) Polycyclic aromatic hydrocarbon-DNA adducts in spontaneously aborted fetal tissue. Carcinogenesis 11: 1673-1675

IARC (1985) Monographs on the evaluation of the carcinogenic risk of chemicals to humans: polycyclic aromatic compounds, bituminous, coal-tars and derived products, shale oils and soots, vol 35. International Agency for Research on Cancer, Lyon, France

Juchau MR (1972) Mechanisms of drug biotransformation reactions in the placenta. Fed Proc 31:48-51

King LC, George M, Gallagher J, Lewtas J (1993) Analysis of 32P-postlabeled DNA adducts by polycyclic aromatic hydrocarbons and nitrated-polycyclic aromatic hydrocarbons in complex mixtures by HPLC and TLC. Chem Res Toxicol 7:503-510

Lewtas J, Mumford J, Everson RB, Hulka B, Wilcosky T, Kozumbo W, Thompson C, George M, Dobias L, Sram R, Li X, Gallagher J (1993) Comparison of DNA

adducts from exposure to complex mixtures in various human tissues and experimental systems. Environ Health Perspect 99:89-97

Manchester DK, Wilson WL, Hsu I-C, Choi J-S, Parker NB, Mann DL, Weston A, Harris CC (1990) Synchronous fluroscence spectroscopic, immunoaffinity chromatographic and ^{32}P-postlabeling analysis of human placental DNA known to contain benzo[a]pyrene diol epoxide adducts. Carcinogenesis 11:553-559

Manchester DK (1981) Range of environmentally responsive monooxygenase activities in human placental microsomes determined by direct fluorescence techniques. Biochem Pharmacol 30:687-692

McLeod S, Rashmi S, Kadulbar F, Lang N (1997) Polymorphisms of CYP 1A1 and GSTM1 influence the in vivo function of CYP 1A2. Mut Res 376:135-142

McRobie DJ, Glover DD, Tracy TS (1996) Regiospecificity of placental metabolism by cytochromes P450 and glutathione S-transferase. Gynecol Obstet Invest 42:154-158

Pasanen M, Pelkonen O (1994) The expression and environmental regulation of P450 enzymes in human placenta. Crit Rev Toxicol 24:211-229

Peterson BA, Chuang CC, Margard WL, Trayser DA (1988) J Air Pollut Control Assoc 81:1-15

Randerath K, Randerath E, Agrawal HP, Gupta RC, Schurdak ME, Reddy MV (1985) Postlabeling methods for carcinogen-DNA adduct analysis. Environ Health Perspect 62:57-65

Smith WA, Gupta RC (1996) Use of a microsome-mediated test system to assess efficacy and mechanisms of cancer chemopreventive agents. Carcinogenesis 17:1285-1290

Vaught JB, Gurtoo HL, Parker NB, LeBoeuf R, Doctor G (1979) Effect of smoking on benzo(a)pyrene metabolism by human placental microsomes. Cancer Res 39:3177-3183

Whyatt RM, Garte SJ, Cosma G, Bell DA, Jedrychowski W, Wahrendorf J, Randall MC, Cooper TB, Ottman R, Tang D (1995) CYP1A1 messenger RNA levels in placental tissue as a biomarker of environmental exposure. Cancer Epidemiol Biomarkers Prev 4:147-153

Williams R, Sparacino C, Peterson B, Bumgarner J, Jungers RH, Lewtas J (1986) Comparative characterization of organic emissions from diesel particles, coke oven mains, roofing tar vapors and cigarette smoke condensate. Intern J Environ Anal Chem 26:27-49