In Vitro Characterization of DNA Adducts Formed by Foundry Air Particulate Matter

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This study is part of an ongoing investigation of biomarkers in iron foundry workers exposed to polycyclic aromatic compounds. Foundry workers with the highest exposures had elevated levels of DNA adducts in their white blood cells in previous studies. The purpose of this study was to characterize the nature of DNA reactive chemicals in foundry air samples through incubating the foundry filter extract with DNA and activation enzymes. Calf thymus DNA was incubated with foundry filter extract and activated by either rat liver activation mixture (S9 mix) or xanthine oxidase. A complex pattern of adducts was observed on thin-layer chromatography (TLC) by the 32P-postlabeling assay. Two selected polycyclic aromatic hydrocarbons (PAHs)—1-NP and anti(±)benzo[a]pyrene-trans-7,8-dihydrodiol-9,10-epoxide (anti(±)BPDE)—DNA adducts—were used as marker compounds in characterizing the postlabeled DNA adducts by TLC combined with high-performance liquid chromatography (HPLC). After an initial separation of DNA adducts by TLC, individual spots were isolated and separated further on HPLC. HPLC analysis and spiking with anti(±)BPDE–DNA standard confirmed the co-migration of the anti(±)BPDE–DNA standard with one PAH adduct formed by the S9 mix-activated DCM extract in calf thymus DNA.

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Introduction

Humans are exposed occupationally and environmentally to polycyclic aromatic compounds including polycyclic aromatic hydrocarbons (PAHs) and nitrated PAHs. PAHs are typical compounds in the atmosphere of coke oven, iron, and steel foundry industries, and nitro-PAHs are found in urban air, coal fly-ash, and diesel engine exhausts (1–8). Both benzo[a]pyrene (B[a]P) and 1-nitropyrene (1-NP) induce mutations in bacteria and tumors in experimental animals (9,10). B[a]P activated by oxidative metabolism leads to the formation of epoxides and diol epoxides that react with DNA to form a binding product with the N2 atom of guanine. Reduction of 1-NP leads to the formation of N-hydroxy-1-aminopyrene that undergoes an acid-catalyzed reaction with DNA and generates the main reaction product of N-(deoxyguanosin-8-yl)1-aminopyrene; two additional 6- and 8-(deoxyguanosin-N2-yl)-1-aminopyrene have also been characterized (11,12). Oxidative metabolism of 1-NP forms 1-NP 4,5-oxide and 1-NP 9,10-oxide, which may form additional DNA binding products (13).

DNA adducts are important biomarkers that provide information about exposure to carcinogens, individual differences in carcinogen metabolism, and DNA repair. In this study PAH and nitro-PAH adducts formed in vitro were analyzed by the 32P-postlabeling assay combined with high-performance liquid chromatography (HPLC). Foundry air samples were collected by a high volume stationary air sampler placed near the iron casting. The organic matter adsorbed to the particles was extracted with dichloromethane (DCM) and used for both chemical and DNA adduct analysis. Calf thymus (CT) DNA was modified in vitro with the DCM extract, and PAH and nitro-PAH were activated by rat liver activation mixture (S9 mix) and xanthine oxidase. Specific 1-NP and anti(±)benzo[a]pyrene-trans-7,8-dihydrodiol-9,10-epoxide [anti(±)BPDE]–DNA adduct standards were used to characterize the in vitro foundry air-derived DNA adducts.

Methods

Foundry Samples

Air samples were collected in an iron foundry in Finland by a high volume stationary air sampler placed 3 to 5 meters away from the iron casting process. The temperature of melted iron was 1380 to 1400°C. The samples were drawn on four 20×20 cm Pallflex filters (type T60A20; Pallflex Products Corp., Putnam, CT). No vapor-phase capture technique was used in this study because the focus was to analyze PAH bound to the particulate matter. Preparation of the extractable organic material (EOM) with DCM and measurements of different PAHs and nitro-PAHs by HPLC and gas chromatography–mass spectrometry (GS–MC) are described elsewhere (14–16). The B[a]P concentration in the composite extract (8.2 ng/mg) was approximately the level of 1-NP (10.9 ng/mg). The B[a]P concentration

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Abbreviations used: anti(±)BPDE, anti(±)benzo[a]pyrene-trans-7,8-dihydrodiol-9,10-epoxide; TLC, thin-layer chromatography; CT, calf thymus; EOM, extractable organic material; S9 mix, rat liver activation mixture; PAHs, polycyclic aromatic hydrocarbons; DCM, dichloromethane; HPLC, high-performance liquid chromatography; GC–MS, gas chromatography–mass spectrometry; B[a]P, benzo[a]pyrene; 1-NP, 1-nitropyrene; WBC, white blood cells.
corresponds to 7 to 41 ng/m³ B[a]P analyzed from the air samples.

Modification of Calf Thymus DNA with Foundry Filter Extract and 1-Nitropyrene

CT DNA (2 mg/ml) was incubated with 100 µl of DCM extract (2.66 mg/ml) and 1-NP (20 µg/ml) under aerobic conditions with S9 mix (0.5 mg/ml) and under anaerobic conditions with xanthine oxidase (0.5 U/ml) and hypoxanthine (0.6 mg/ml) for 4 hr at 37°C as previously described (14-16).

Anti(±)Benzo[a]pyrene-trans-7,8-dihydrodiol-9,10-epoxide-DNA Standard Preparation

The anti(±)BPDE-DNA standard was prepared in 100 mM Tris–HCl buffer, pH 7.0, and incubated at room temperature for 16 hr with CT DNA (1 mg/ml) with 0.1 volume of anti(±)BPDE (1 mg/ml). The reaction mixture was extracted twice with water-saturated butanol and with diethylether.

32P-Postlabeling Analysis

DNA was analyzed by the butanol extraction and nuclease P1-enhanced postlabeling assay according to Gupta (17) and Reddy and Randerath (18). The labeled DNA adducts were resolved on 10 x 10 or 20 x 20 cm polyethylene imine (PEI) cellulose thin-layer chromatography (TLC) plates (Macherey-Nagel, Düren, Germany) using a standard solvent system previously described (19), with the exception that 1.7 M sodium phosphate, pH 6, was used for D5. The radioactive areas of adducts were located by autoradiography using intensifying screens, and adduct levels were calculated from the amount of radioactivity on the chromatograms, DNA, and the specific activity of [γ-32P]ATP used for the labeling (18).

HPLC Analysis

HPLC separation was carried out according to Pfau and Phillips (20) and King et al. (16) with slight modifications. The Varian Model 5500 (Varian, Walnut Creek, CA) was used for HPLC, and radioactivity was determined with the flow-through scintillation counter Model IC/CR Flo-One β (Radiomatic Instruments & Chemical Co., Inc., Tampa, FL) using 2.5 ml/cell and a scintillator flow rate of 1.8 ml/min. The radioactive spots were eluted from TLC plates by extracting in 400 µl of 4 M pyridinium formate, pH 4.0, for 18 hr. The adduct residues were separated in an Inertil ODS-2 column (5 µm particle size, 2.1 mm x 150 mm; GL Sciences Inc.) with a flow rate of 0.45 ml/min. The solvent system used 0.3 M NaH₂PO₄/H₃PO₄, pH 2.5, and 100% MeOH. HPLC samples were separated with two gradient systems: gradient A was increased with MeOH from 10 to 50% in 0 to 20 min, 50 to 90% in 20 to 40 min, 90 to 70% in 40 to 50 min, 70 to 10% in 50 to 53 min, and to 10% in 70 min; gradient B was increased with MeOH from 10 to 45% in 0 to 15 min, 45 to 48% in 15 to 60 min, 48 to 90% in 60 to 80 min, 90 to 10% in 80 to 81 min, and to 10% in 90 min.

Figure 1. Autoradiographs of 32P-postlabeled human DNA. CT DNA was modified in vitro with DCM extract, and PAH and nitro-PAH were activated by the S9 mix and xanthine oxidase. A and B, 1-NP-DNA adducts analyzed by the postlabeling assay enhanced by nuclease P1 and butanol extraction, respectively. C, in vitro-prepared anti(±)BPDE-DNA adduct standard. D and E, Diagonal radioactive zone of in vitro-modified CT DNA analyzed on 10 x 10 cm and 20 x 20 cm TLC plates, respectively (1-4 are migrating DNA adducts). F, 32P-postlabeled DNA digest from WBC of an exposed foundry worker (black circle shows the area excised for the quantitation of adducts; adducts 3 and 4 were not detected). All samples shown in B through F were analyzed by the butanol extraction. Autoradiography was carried out at room temperature for 1 min for A through C and 5 hr for D and E and at ~70°C for 3 days for F.
Results and Discussion

During the 1980s, a series of biomarker studies was initiated in iron foundry workers. DNA adducts were analyzed in white blood cells (WBC) of these workers by Phillips et al. (21) using a $^{32}$P-postlabeling assay and by Santella et al. (22) using an enzyme-linked immunosorbent assay. In this study, B[a]P levels in the workplace atmosphere ranged from 7 to 41 ng/m$^3$, which are about 50-fold lower than the B[a]P levels of 2 to 3 µg/m$^3$ measured earlier (1979–1980) in the same foundry (23). The 1990 PAH measurements were determined by personal monitors worn by the workers; the B[a]P levels ranged from 2 to 60 ng/m$^3$ (22).

The concentration of B[a]P in the compositive extract of foundry air particles was 8.2 ng/mg EOM. 1-NP was also quantitated in this extract at the concentration comparable to B[a]P (10.9 ng/mg). Figures 1A and 1B show the autoradiographs from the 1-NP–DNA standard using both nuclease P1 and butanol extraction. Figure 1C shows the anti(±)BPDE–DNA adduct standard analyzed by butanol extraction. 1-NP was only detected by the butanol extraction-enhanced $^{32}$P-postlabeling assay. The DNA adducts formed from a foundry sample show a diagonal radioactive zone of adducts obtained on both 10 × 10 and 20 × 20 cm TLC from the in vitro with DCM extract-modified CT DNA using the butanol extraction-enhanced $^{32}$P-postlabeling assay (Figures 1D, 1E). The chromatography on the 20 × 20 cm TLC plates showed separation more clearly (Figure 1E). Foundry DNA adducts formed by xanthine oxidase were barely detectable (data not shown). In the presence of S9 mix, relatively high levels of several DNA adducts were observed. DNA adducts detected by the butanol extraction from a foundry worker's WBC DNA showed low levels of radioactivity, and no DNA adducts were detected on the area of 1-NP (Figure 1F). However, DNA adducts migrating close to the origin could correspond to adducts 1 and 2 detected on 10 × 10 cm TLC plates from the in vitro foundry DNA samples (Figure 1D). Further migrating DNA adducts 3 and 4 (Figure 1D) were not detected in foundry workers' WBC DNA.

TLC combined with HPLC was used in this study to characterize PAH standards and adducts formed in CT DNA incubated with foundry filter DCM extract. Anti(±)BPDE– and 1-NP–DNA adducts were eluting close together when gradient A was used in HPLC analysis (Figure 2A);
however, the modified gradient B separated PAH standards distinctly (Figure 2B). Gradient A, however, was used further in this study because no 1-NP adducts were detected on TLC. The anti(α)BPDE–DNA adduct standard was used as a marker compound in HPLC analysis in characterizing PAH adducts formed in CT incubated with DCM extract (Figures 3A–H). Figure 3A, 3C, 3E, and 3G show the HPLC analysis of four adducts obtained on both 10×10 or 20×20 cm TLC plates (Figures 1D, 1E). These in vitro adduct residues were analyzed first by HPLC; the same four residues were spiked before the HPLC with the anti(α)BPDE–DNA adduct standard (Figures 3B, 3D, 3F, 3H). The main peak of adduct 2 is most probably derived from B[a]P based on co-migration with the anti(α)BPDE–DNA standard (Figures 3C, 3D). The development of the methodology in analyzing nitro-PAH–DNA adducts in exposed humans by these two techniques still needs to be improved; however, promising results have been reported in analyzing PAH and nitro-PAH adducts from in vivo DNA (16,24–26). This study confirms that the 32P-postlabeling method combined with HPLC is an applicable method for the characterization of PAH adducts in in vivo DNA exposed to complex mixtures of PAHs. The ultimate objectives of these studies are to characterize and identify specific PAH– and nitro-PAH–DNA adducts in human cells from exposed foundry workers.

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