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Formation of Stable DNA Adducts and Apurinic Sites upon Metabolic Activation of Bay and Fjord Region Polycyclic Aromatic Hydrocarbons in Human Cell Cultures

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Carcinogenic polycyclic aromatic hydrocarbons (PAH), such as benzo[*a*]pyrene (B[*a*]P), 7,12-dimethylbenz[*a*]anthracene (DMBA), and dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P), are metabolically activated to electrophilically reactive bay or fjord region diol epoxides that bind to the exocyclic amino groups of purine bases in DNA to form stable adducts. In addition, it has been reported that these PAH can be enzymatically oxidized to yield radical cations that form apurinic (AP) sites in DNA via depurinating adducts. The formation of stable adducts and AP sites in DNA of human cells exposed to PAH was examined in cytochrome P450 (P450)-expressing mammary carcinoma MCF-7 cells and in leukemia HL-60 cells, which display a high peroxidase but no P450-mediated activity, after exposure to these PAH. Stable DNA adducts were assessed by ^{33}P-postlabeling/HPLC analysis, and the induction of AP sites in DNA was analyzed by an aldehyde reactive probe (ARP) and a slot blot method. After exposure for 4 h, the levels of stable DNA adducts were comparable in MCF-7 cells treated with B[*a*]P and DMBA, but significantly lower than those observed in MCF-7 cells treated with the stronger carcinogen DB[*a*,*l*]P. While the levels of stable adducts increased more than 10-fold (B[*a*]P and DMBA) or 100-fold (DB[*a*,*l*]P) after exposure for 24 h, the levels of AP sites remained low after both treatment periods. Thus, the levels of stable adducts were approximately 5-fold higher than the levels of AP sites after treatment with B[*a*]P or DMBA and more than 100-fold higher in cells exposed to DB[*a*,*l*]P for 24 h. None of these carcinogenic PAH formed detectable levels of stable DNA adducts or AP sites in HL-60 cells. The results demonstrate that metabolic activation of B[*a*]P, DMBA, and DB[*a*,*l*]P is catalyzed by P450 enzymes leading to diol epoxides that form predominantly stable DNA adducts but only low levels of AP sites.

Introduction

Numerous polycyclic aromatic hydrocarbons (PAH)[1] are carcinogenic environmental pollutants that require metabolic activation to exert their genotoxic effects (*1*−*3*). Two major pathways for metabolic activation of PAH have been described: (a) diol epoxide formation via successive epoxidations catalyzed by cytochrome P450 (P450) monooxygenases and an intermediate hydrolysis step catalyzed by microsomal epoxide hydrolase (*3*−*5*) and (b) enzymatically catalyzed one-electron oxidation leading to reactive radical cation intermediates (*6*).

Tumor induction studies with many PAH, including dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P), 7,12-dimethylbenz[*a*]anthracene (DMBA), and benzo[*a*]pyrene (B[*a*]P) (Scheme 1), demonstrated that DB[*a*,*l*]P is a significantly stronger tumor initiator than any other PAH tested in mouse skin and rat mammary gland. In rodent tumor models, the order of carcinogenic potencies of these PAH was as follows: DB[*a*,*l*]P ≫ DMBA > B[*a*]P (*7*−*10*). Investigations of DNA adduct formation by these PAH helped to define the relationship between metabolic activation, DNA binding, and carcinogenic potency. Several laboratories have characterized the DNA adducts formed by the bay region PAH B[*a*]P in mammalian cells and have identified (+)-*anti*-B[*a*]P-7,8-diol 9,10-epoxide (BPDE)−2′-deoxyguanosine (dG) as the major DNA adduct that is formed (*11*−*13*). Dipple and co-workers demonstrated

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[1] Abbreviations: AP, apurinic; ARP, aldehyde reactive probe; B[*a*]P, benzo[*a*]pyrene; BPDE, B[*a*]P-7,8-diol 9,10-epoxide; dA, 2′-deoxyadenosine; DB[*a*,*l*]P, dibenzo[*a*,*l*]pyrene; dG, 2′-deoxyguanosine; DHFR, dihydrofolate reductase; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMBADE, DMBA-3,4-diol 1,2-epoxide; D-MEM/F-12, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12; DMS, dimethyl sulfate; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P450, cytochrome P450; PAH, polycyclic aromatic hydrocarbon(s); PBS, phosphate-buffered saline.

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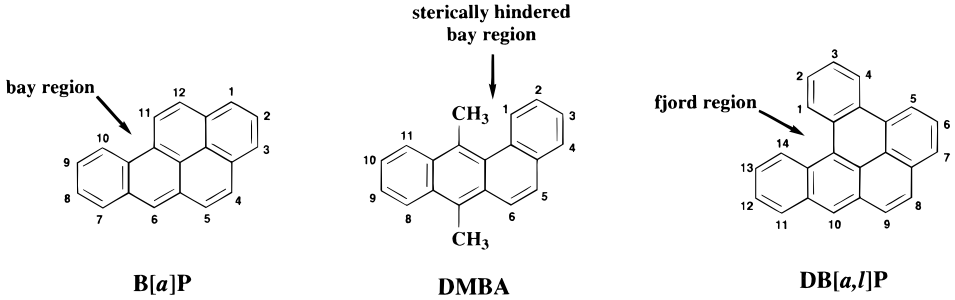
Scheme 1. Structures of the Three Carcinogenic Polycyclic Aromatic Hydrocarbons (PAH), Benzo[*a*]pyrene (B[*a*]P), 7,12-Dimethylbenz[*a*]anthracene (DMBA), and Dibenzo[*a,l*]pyrene (DB[*a,l*]P), Used in This Study[a]



[a] Arrows indicate the three different regions at which diol epoxides are formed for various carcinogenic PAH.

that DMBA is metabolically activated to both *syn*- and *anti*-DMBA-3,4-diol 1,2-epoxides (DMBADEs) which bind extensively to both dG and 2′-deoxyadenosine (dA) residues in DNA of mouse embryo cells (*4*, *14*, *15*). Analysis of DNA adducts after exposure of human mammary epithelial cell cultures to DMBA revealed that human cells catalyze predominantly the formation of *syn*-DMBADE−dA adducts (*16*). Extensive binding to dA residues in DNA has also been reported for the highly carcinogenic fjord region diol epoxides of benzo[*c*]phenanthrene and benzo[*g*]chrysene, suggesting that formation of high levels of dA adducts may be related to the high tumorigenic activity of PAH such as DMBA (*14*, *17*, *18*).

In contrast to the stable adducts described above, Cavalieri and co-workers have reported that the fjord region PAH DB[*a,l*]P (Scheme 1) forms mainly depurinating adducts (84%) upon metabolic activation by rat liver microsomes (*19*). They also reported that 99% of all DMBA−DNA adducts in vitro and in mouse skin (*20*−*22*) and 68% of the B[*a*]P−DNA adducts in vitro and 71% of those in mouse skin were unstable and subsequently depurinated to form apurinic (AP) sites (*23*−*25*). On the basis of these results, the authors have proposed that these PAH are predominantly activated by one-electron oxidation to form depurinating DNA adducts that result in AP sites and that only a small proportion of total DNA binding is due to the formation of stable adducts from reactive diol epoxides (*6*, *26*). Depurinating DNA adducts of DMBA or B[*a*]P are reported to form at least a 10-fold higher level of AP sites compared to the level formed in cells by spontaneous depurination (about 2000 AP sites over the course of 4 h) (*27*). Therefore, Cavalieri and coworkers hypothesized that AP sites generated by depurinating PAH−DNA adducts in excess of the repair capacity of the cell (*6*, *26*, *27*) or the repair activity of the cell itself (*28*) may lead to transforming mutations and be responsible for tumor initiation in vivo.

DB[*a,l*]P forms high amounts of stable DNA adducts upon metabolic activation in human mammary carcinoma MCF-7 cells (*3*). No AP sites were detected in DNA from DB[*a,l*]P-treated MCF-7 cells by a Southern blot technique for analysis of AP sites in the dihydrofolate reductase (DHFR) gene (*29*). In the study presented here, the relative proportions of stable DNA adducts and AP sites formed by B[*a*]P, DMBA, and DB[*a,l*]P were investigated in human mammary carcinoma MCF-7 and human leukemia HL-60 cells. In contrast to MCF-7 cells, which exhibit a high P450-mediated metabolic activation capacity for PAH, human leukemia HL-60 cells possess high intracellular peroxidase activity but no detectable P450 activity responsible for activation of PAH to diol epoxides (*30*−*32*). The ^{33}P-postlabeling technique with HPLC analysis was used to measure the extent of formation of stable PAH−DNA adducts. Changes in the levels of AP sites as a result of the formation of depurinating DNA adducts were determined by a slot blot method utilizing a highly sensitive aldehyde reactive probe (ARP) reagent (*33*) which binds to aldehyde groups in ring-opened sugar residues formed at AP sites. The combination of both methods allowed a quantitative analysis and comparison of the relative proportions of stable adducts and AP sites formed in DNA of cells upon P450- or peroxidase-mediated metabolic activation of these PAH.

Experimental Procedures

Caution: *All PAH used in this study are chemical carcinogens and should be handled according to the guidelines of the National Cancer Institute.*

Chemicals. B[*a*]P, DMBA, and DB[*a,l*]P were purchased from Chemsyn Science Laboratories (Lenexa, KS). The ARP reagent was synthesized as previously described by Kubo et al. (*33*) and then checked by application in an enzyme-linked immunosorbent assay (ELISA) to verify its ability to detect known numbers of AP sites in DNA of Chinese hamster ovary cells (*33*, *34*).

Cell Culture and Treatment of Cells. MCF-7 cells were grown in 175 cm^2 flasks in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (1:1 D-MEM/F-12 mixture) supplemented with 10% fetal calf serum (FCS), 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and antibiotics (200 units/mL penicillin, 200 μg/mL streptomycin, and 25 μg/mL ampicillin). The cells were maintained and treated at 37 °C in a humidified 5% CO_2/95% air atmosphere. Cells were refed fresh medium after they covered >90% of the surface area of the culture dish. Twenty-four hours later, the cells were treated with 1 or 2 μM B[*a*]P, DMBA, or DB[*a,l*]P for 4 or 24 h. Control cultures were treated with the solvent (Me_2SO) alone. The cells were harvested by trypsinization with 0.05% trypsin/EDTA and washed with PBS. DNA was isolated by treatment with RNase, proteinase K, and phenol followed by chloroform/isoamyl alcohol (24:1) extractions as described previously (*29*, *35*, *36*). The DNA was precipitated with 2 volumes of ethanol and dissolved in TE buffer [10 mM Tris/1 mM EDTA (pH 8.0)]. DNA concentrations in the preparations were determined spectrophotometrically at 260 nm.

The human promyelocytic leukemia HL-60 cell line was maintained in 175 cm^2 flasks with D-MEM/F-12 medium, supplemented with 10% FCS, HEPES buffer, and antibiotics, resuspended at a density of ~10^7 cells/100 mL, and then treated with 2, 6, 10, or 30 μM B[*a*]P, DMBA, or DB[*a,l*]P for 4 h. Cells were harvested by centrifugation at 3000*g* for 10 min and

washed with phosphate-buffered saline {PBS [2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, and 8.1 mM Na_2HPO_4 (pH 7.0)]}, and DNA was isolated as described above.

Detection of AP Sites with the ARP-Slot Blot Assay. AP sites were detected using the ARP-slot blot assay described by Nakamura et al. (*37*) with the minor modifications described below. Fifteen micrograms of DNA obtained from PAH-treated cells was dissolved in 100 μL of PBS, mixed with 50 μL of ARP reagent, and incubated at 37 °C for 15 min. DNA was precipitated by adding ice-cold ethanol, washed with 70% ethanol, and redissolved in TE buffer at a concentration of 3 μg/100 μL. After denaturation at 100 °C for 5 min, DNA samples were immediately chilled on ice, and an equal volume of 2 M ammonium acetate was added. Three micrograms of denaturated DNA was then immobilized on a BAS-85 nitrocellulose membrane using a Minifold II vacuum filter device (Schleicher & Schuell, Keene, NH). After the slots were rinsed with 200 μL of 1 M ammonium acetate, the membrane was soaked with a solution containing 0.75 M NaCl and 0.075 M trisodium citrate at 37 °C for 20 min and then dried at 80 °C for 3 h. The membrane was blocked in 10 mL of Tris-NaCl buffer [20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 0.5% casein, 0.25% bovine serum albumin, and 0.1% Tween 20] at room temperature for 1 h. The membrane was then incubated in a fresh Tris-NaCl buffer solution containing streptavidin-conjugated horseradish peroxidase (Gibco BRL, Gaithersburg, MD) at room temperature for 40 min. After the membrane was rinsed with washing buffer (0.26 M NaCl, 1 mM EDTA, 20 mM Tris-HCl, and 0.1% Tween 20), the enzymatic activity on the membrane was visualized using enhanced chemiluminescence detection (Amersham Life Science Inc., Arlington Heights, IL). The membrane was exposed to X-ray film (FUJI Medical Systems Inc., Stanford, CA) for 30 s, and the developed film was analyzed using a scanning densitometer (Molecular Dynamics, Sunnyvale, CA) with ImageQuaNT Software (Molecular Dynamics). Quantitation of AP sites was based on comparison to a calibration curve generated with dimethyl sulfate (DMS)-treated DNA of Chinese hamster ovary (CHO) cells in which the level of AP sites had been determined using a Southern blot method for the DHFR gene as previously described (*29*).

Stable DNA Adduct Analysis by ^{33}P-Postlabeling. Ten micrograms of DNA from MCF-7 or HL-60 cells treated with the PAH was ^{33}P-postlabeled using the nuclease P1 and prostatic acid phosphatase protocol and Sep-Pak C_{18} column chromatography as previously described (*29*). Separation of the ^{33}P-labeled PAH−DNA adducts was accomplished by reverse-phase HPLC utilizing a 5 μm Ultrasphere C_{18} column (4.6 mm × 25 cm; Beckman Instruments Inc., St. Louis, MO) and an on-line radioisotope detector (Radiomatic Flo-ONE BETA, Packard Instruments, Downers Grove, IL). The DMBA−DNA adducts were separated using 0.05 M ammonium phosphate buffer (pH 5.5) that contained 20 mM tetrabutylammonium phosphate (solvent A) and 100% methanol (solvent B) at a flow rate of 1 mL/min. The gradient that was used was as follows: 50% B for 25 min, 50 to 57% B over the course of 70 min, 57 to 62% B over the course of 20 min, 62 to 43% B over the course of 20 min, and 43% B for 20 min. The B[*a*]P−DNA adducts were separated using 0.1 M ammonium phosphate buffer (pH 5.5) (solvent A) and 100% methanol (solvent B) and the following gradient: 44% B for 10 min, 44 to 49% B over the course of 50 min, 49 to 58% B over the course of 15 min, and 58% B for 45 min. The DB[*a*,*l*]P−DNA adducts were separated using 0.1 M ammonium phosphate buffer (pH 5.5) (solvent A) and 10% acetonitrile/90% methanol (solvent B) and the following gradient: 44 to 49% B over the course of 40 min, 49 to 55% B over the course of 60 min, and 55 to 65% B over the course of 20 min. The total level of stable PAH−DNA adducts was calculated on the basis of a [^{3}H]B[*a*]PDE−DNA standard of known modification which was included in each of the postlabeling experiments.

Statistical Analysis. Statistical analyses of all experiments were conducted using the Student's *t* test and the one-way analysis of variance (ANOVA) for the number of AP sites and stable DNA adducts.

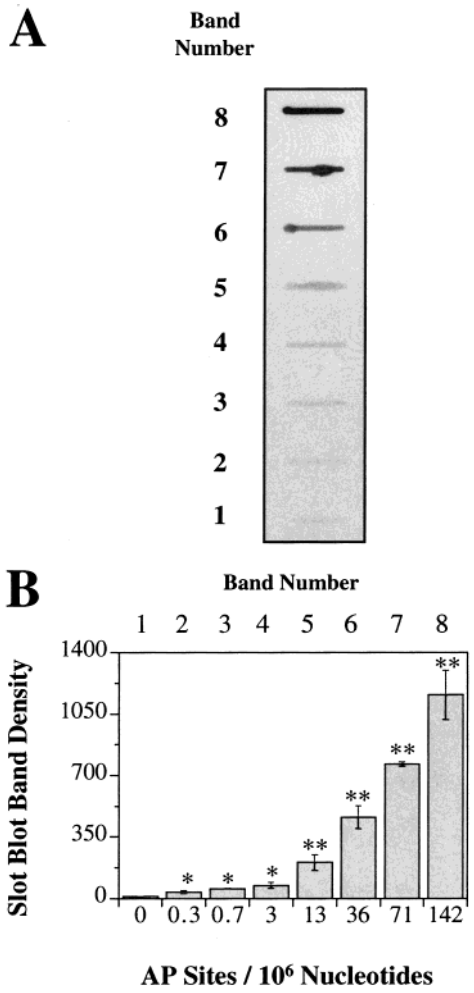


Figure 1. Standard curve for the detection of AP sites in DNA using the ARP-slot blot assay. DNA containing known numbers of AP sites was serially diluted with isolated DNA of CHO cells and then incubated with the ARP reagent (DNA containing zero AP sites above the background was entirely unmodified DNA of CHO cells). (A) X-ray film of a blot containing 3 μg of DNA per slot loaded on a nitrocellulose membrane. The level of AP sites in each band is given in panel B. (B) Intensities of the slots shown in panel A were measured by densitometry. The slot numbers are indicated at the top. The data represent the mean of four independent determinants; the bars represent SD. Values significantly different from the control value: $P < 0.01$ and $P < 0.005$.

Results

MCF-7 and HL-60 cells were treated with different doses of B[*a*]P, DMBA, and DB[*a*,*l*]P for 4 and 24 h so the levels of stable DNA adducts and AP sites could be measured. The sensitivity of the ARP-slot blot method used in this study for assessment of AP sites (*37*, *38*) was compared to that of the procedure based on Southern blot analysis used in previous studies by analyzing the same standard of methylated DNA obtained from CHO cells that contained 142 induced AP sites per 10^6 nucleotides (*29*). A typical X-ray film obtained after visualization of AP sites in serially diluted DNA from CHO cells is shown in Figure 1A. The values obtained by quantitation by densitometry (Figure 1B) demonstrate a positive correlation between the number of AP sites and the slot band intensity on the nitrocellulose membrane. The increase

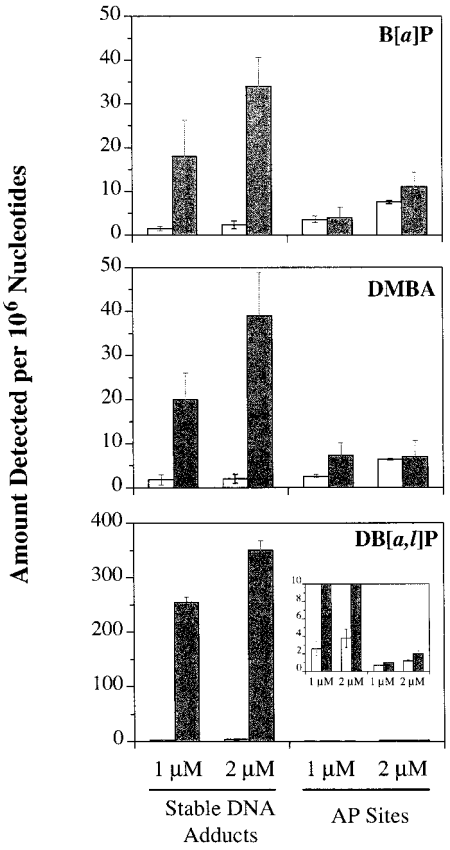


Figure 2. Analysis of stable DNA adducts and AP sites present in DNA of MCF-7 cells exposed to 1 or 2 μM B[*a*]P, DMBA, or DB[*a,l*]P for 4 h (white bars) and 24 h (gray bars). AP sites and stable adducts in DNA were analyzed using the ARP-slot blot assay and the ^{33}P-postlabeling/HPLC technique as described in Experimental Procedures. The level of AP sites in DNA of solvent-treated control samples ranged from 0.6 to 1.2 AP sites per 10^6 nucleotides. The data represent the mean of three independent determinations; the bars represent SD. Statistical analyses of the data indicated that the following comparisons contained values which are significanly different: $P < 0.0001$, AP sites induced by B[*a*]P or DMBA vs control after 4 and 24 h, AP sites induced by B[*a*]P vs DMBA at 2 μM after 4 h, AP sites induced by DB[*a,l*]P vs B[*a*]P or DMBA at 2 μM after 4 h, stable adducts induced by all PAH vs AP sites induced by all PAH at all doses after 24 h, and stable adducts induced by DMBA vs AP sites induced by DMBA at 2 μM after 24 h; $P < 0.0005$, AP sites induced by DB[*a,l*]P at 2 μM after 4 h, stable adducts induced by B[*a*]P vs AP sites induced by the respective PAH at 2 μM after 24 h, and stable adducts induced by DMBA vs AP sites induced by DMBA at 1 μM after 24 h; $P < 0.001$, stable adducts induced by B[*a*]P or DMBA vs AP sites induced by the respective PAH at 2 μM after 24 h; $P < 0.01$, AP sites induced by B[*a*]P vs DMBA at 2 μM after 4 h; $P < 0.05$, AP sites induced by B[*a*]P vs DMBA at 2 μM after 4 h, AP sites induced by DB[*a,l*]P vs B[*a*]P or DMBA at all doses after 24 h, stable adducts induced by DB[*a,l*]P vs AP sites induced by DB-[*a,l*]P at all doses after 4 h, and stable adducts induced by B[*a*]P vs AP sites induced by B[*a*]P at 1 μM after 24 h. Other sets of comparisons were not significantly different ($P > 0.05$).

in slot intensity compared to that of the control DNA is statistically significant for DNA samples containing as few as three AP sites per 10^7 nucleotides ($P = 0.0052$).

The levels of stable DNA adducts and AP sites detected in DNA of MCF-7 cells after exposure to 1 or 2 μM B[*a*]P, DMBA, or DB[*a,l*]P for 4 and 24 h are shown in Figure 2. The numbers of AP sites in cellular DNA after exposure to PAH were determined on the basis of the slot band intensities observed for methylated DNA of CHO cells (Figure 1). DNA from MCF-7 cells after treatment with the solvent (Me_2SO) gave very low slot band

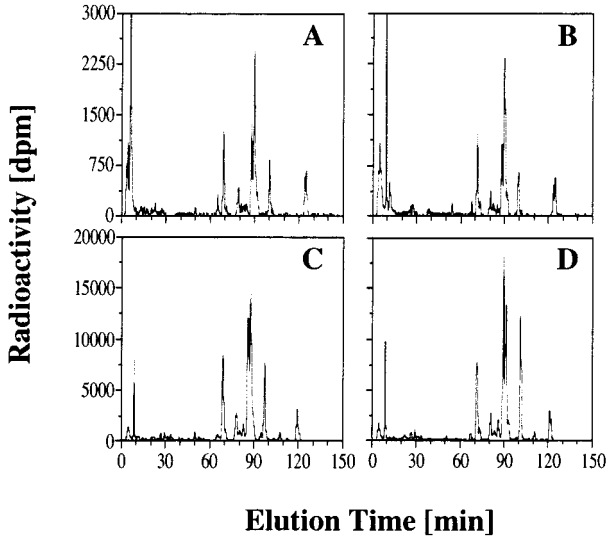


Figure 3. HPLC elution profiles of the ^{33}P-labeled DMBA−DNA adducts formed in MCF-7 cells after incubation for 4 h with (A) 1 or (B) 2 μM DMBA or after incubation for 24 h with (C) 1 or (D) 2 μM DMBA. All adduct peaks eluting between 60 and 130 min have been previously identified as DMBADE−dG or −dA adducts (*39*).

intensities. Statistically significant increases in the number of AP sites were observed in DNA isolated from cells after treatment with DMBA or B[*a*]P ($P < 0.05$ for DMBA and $P < 0.005$ for B[*a*]P) (Figure 2). The levels of AP sites in DNA obtained from MCF-7 cells exposed to 1 or 2 μM B[*a*]P for 4 h were not statistically different than those obtained from the same doses of DMBA ($P > 0.1$). However, the level of AP sites in DNA of cells treated with 2 μM B[*a*]P for 24 h was significantly higher than that found after exposure to the same dose of DMBA ($P < 0.01$). In contrast, the number of AP sites in DNA isolated from cells after treatment with DB[*a,l*]P did not differ significantly from that of the solvent control ($P > 0.1$). The levels of AP sites in DNA from DB[*a,l*]P-treated cells were significantly lower than those observed after treatment with B[*a*]P or DMBA ($P < 0.05$) (Figure 2).

Stable PAH−DNA adducts formed in MCF-7 cells during treatment with B[*a*]P, DMBA, or DB[*a,l*]P were analyzed using ^{33}P-postlabeling and HPLC techniques. The HPLC elution profiles of the DMBA− and B[*a*]P−DNA adducts are shown in Figures 3 and 4. MCF-7 cells treated with 1 or 2 μM DMBA for 4 or 24 h contained two major DNA adducts which eluted at 90 min (Figure 3) and which have been previously identified as *syn*-DMBADE−dA and *anti*-DMBADE−dG adducts (*39*). The HPLC profiles also contained several minor peaks which correspond to *anti*-DMBADE−dG (70 min), *anti*-DMBADE−dA (100 min), and *syn*-DMBADE−dA adducts (120 min). MCF-7 cells treated with B[*a*]P at both doses and treatment times (Figure 4) contained one major adduct peak which has been previously identified as the *anti*-BPDE−dG adduct (*12*, *13*, *40*). The HPLC profiles of DNA adducts formed in MCF-7 cells after exposure to DB[*a,l*]P were reported previously (*29*).

The levels of stable DNA adducts detected in MCF-7 cells after treatment with 1 or 2 μM B[*a*]P, DMBA, or DB[*a,l*]P for 4 or 24 h are shown in Figure 2. After treatment for 4 h, the number of stable DNA adducts in cells treated with DB[*a,l*]P was approximately twice as high as that in cells treated with DMBA or B[*a*]P (Figure 2). After treatment for 24 h, this difference increased to

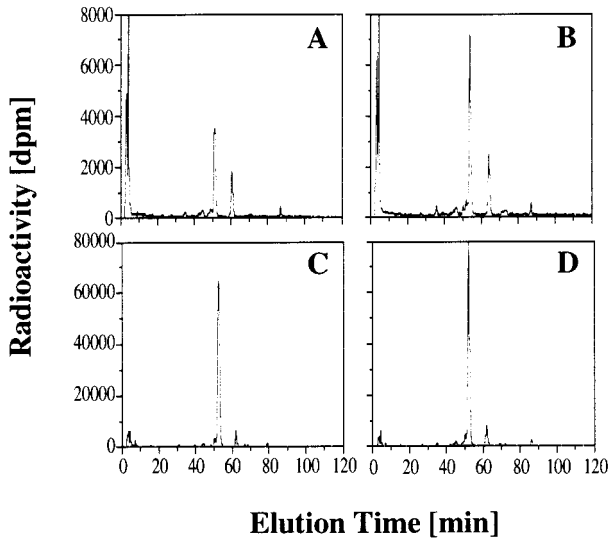


Figure 4. HPLC elution profiles of the ^{33}P-labeled B[*a*]P−DNA adducts formed in MCF-7 cells after incubation for 4 h with (A) 1 or (B) 2 μM B[*a*]P or after incubation for 24 h with (C) 1 or (D) 2 μM B[*a*]P. The major adduct peak eluting around 50 min has been identified as the (+)-*anti*-BPDE−dG adduct, whereas the minor peak at 60−65 min consisted of an isomeric *syn*-BPDE−dG adduct (*12*).

an approximately 8-fold higher level of DNA binding of DB[*a*,*l*]P compared to that of DMBA or B[*a*]P (Figure 2). B[*a*]P and DMBA formed similar levels of stable DNA adducts in MCF-7 cells at both doses ($P > 0.1$). The levels of AP sites detected in DNA after exposure to 1 μM B[*a*]P or DMBA for 4 h were comparable ($P > 0.1$) (Figure 2). However, after exposure to 2 μM PAH, DNA from B[*a*]P-treated cells contained significantly more AP sites than DNA from DMBA-treated cells ($P < 0.01$). After treatment for 24 h, the levels of stable DMBA− and B[*a*]P−DNA adducts increased more than 10-fold, whereas the level of AP sites remained constant ($P < 0.0001$). On the basis of the levels of stable DNA adducts and AP sites in DNA of MCF-7 cells, the relative proportions of stable adducts after treatment with B[*a*]P and DMBA were in the range of 70%. In DB[*a*,*l*]P-treated cells, the proportion of stable adducts was greater than 99%.

The levels of AP sites detected in HL-60 cells after treatment with four concentrations of B[*a*]P, DMBA, or DB[*a*,*l*]P for 4 h are shown in Figure 5. Control DNA obtained from solvent-treated HL-60 cells (Figure 5) contained approximately 10 AP sites per 10^6 nucleotides, a higher level of AP sites than that observed in DNA obtained from solvent-treated MCF-7 cells (approximately one AP site per 10^6 nucleotides). The number of AP sites in HL-60 cells did not increase significantly after exposure to B[*a*]P, DMBA, or DB[*a*,*l*]P, even with extremely high doses of these compounds ($P > 0.1$; Figure 5). HPLC profiles of ^{33}P-postlabeled DNA obtained from HL-60 cells after exposure to B[*a*]P, DMBA, or DB[*a*,*l*]P did not contain any detectable adduct peaks at times up to 24 h (data not shown). HL-60 cells were also incubated with 10 μM [^{3}H]B[*a*]P to determine whether the peroxidase activity present in HL-60 cells was capable of metabolizing PAH to water-soluble metabolites. Analysis of media samples extracted by a two-stage chloroform/methanol/water procedure (*41*, *42*) after exposure of HL-60 cells for periods of 2−48 h demonstrated that no conversion of [^{3}H]B[*a*]P to water-soluble metabolites was observed (data not shown).

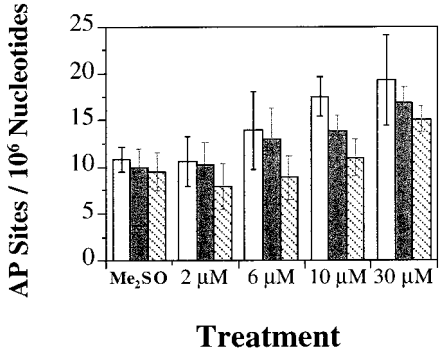


Figure 5. Levels of AP sites detected in HL-60 cells after treatment with 0 (Me_2SO only), 2, 6, 10, or 30 μM B[*a*]P (white bars), DMBA (gray bars), or DB[*a*,*l*]P (hatched bars) for 4 h. The number of AP sites in cellular DNA was measured using the ARP-slot blot assay as described in Experimental Procedures. The data represent the mean of three independent determinations; the bars represent SD. Statistical analyses of the data indicated that all sets of comparisons were not significantly different ($P > 0.1$).

Discussion

Understanding the mechanisms of PAH activation and their role in determining the differences in carcinogenic potency among PAH such as B[*a*]P, DMBA, and DB[*a*,*l*]P is important for assessing the risk PAH pose to humans as a population and as individuals. Tumor incidence values obtained after treatment of different species and tissues of rodents revealed that DB[*a*,*l*]P is a more potent carcinogen than DMBA which exhibits greater potency than B[*a*]P (*8*, *9*). Extensive evidence shows that these PAH exert their carcinogenic activity via metabolic activation to electrophilically reactive bay or fjord region diol epoxides that covalently bind to DNA to form stable adducts which lead to the induction of mutations in critical genes and subsequently to neoplastic transformation of the target cells (*3*). Another proposed pathway of metabolic activation involves a one-electron oxidation step catalyzed by P450 or peroxidase enzymes which yields radical cation intermediates which bind to DNA to form unstable depurinating adducts. The loss of these unstable DNA adducts by depurination may lead to the generation of AP sites (*6*, *26*, *27*) and subsequently to mutations if their numbers exceed the DNA repair capacity of the affected cell (*43*).

Investigations of carcinogen−DNA interactions have demonstrated that stable DNA adduct formation can lead to mutation through misincorporation of nucleotides or by causing deletions (*44*). Generation of such DNA lesions at certain critical locations in the genome (e.g., proto-oncogenes of the *ras* family) may subsequently result in tumor initiation. Cell culture studies with diol epoxides of PAH have demonstrated that formation of stable DNA adducts can lead to induction of mutations (*45*). Analysis of mouse skin tumors obtained after topical application of B[*a*]P revealed a high frequency of dG mutations at codons 12 and 13 of the cellular protooncogene Ha-*ras* (*27*, *46*−*48*). High frequencies of G → T transversions in codon 12 and A → T transversions in codon 61 of cellular Ha-*ras* detected in vivo after exposure to B[*a*]P and DMBA, respectively, are consistent with the extensive and preferential binding of metabolically generated diol epoxide derivatives of these respective PAH to dG or dA residues in DNA (*49*−*52*). Induction of lung tumors in mice by DB[*a*,*l*]P and subsequent analysis of the cellular

Ki-*ras* gene demonstrated that a positive correlation exists between the level of stable DNA adducts formed by diol epoxides and the frequency of mutations in this gene (*53*). However, Chakravarti et al. (*27*, *28*) proposed that A → T transversions in the Ha-*ras* gene detected in DB[*a*,*l*]P-induced mouse skin papillomas could have resulted from AP sites formed by depurinating DNA adducts (*27*).

The study presented here was designed to elucidate the relative proportions of stable adducts and AP sites formed in DNA of mammalian cells in culture after exposure to three potent carcinogens, B[*a*]P, DMBA, and DB[*a*,*l*]P. No evidence for AP site induction in DNA of cells exposed to DB[*a*,*l*]P was found in previous studies using an alkaline Southern blot method for analysis of a restriction fragment of the DHFR gene (*29*) or an ELISA-like ARP assay (*38*). In the study presented here, a modified ARP-slot blot assay was used to increase the sensitivity of AP site detection to about three AP sites per 10^7 nucleotides (Figure 1). Even with this greater sensitivity, DNA obtained from cells treated with DB-[*a*,*l*]P showed no detectable elevation in levels of AP sites compared to that of the solvent-treated control (Figure 2). The high levels of stable adducts, about 250−350 per 10^6 nucleotides, formed by the metabolically generated 11,12-diol 13,14-epoxides of this PAH during a treatment period of 24 h (Figure 2), together with high mutagenic activities of DB[*a*,*l*]P, its 11,12-diol, and its 11,12-diol 13,14-epoxides previously observed in Chinese hamster V79 cells (*54*−*56*), indicate that P450-catalyzed transformation of DB[*a*,*l*]P to fjord region diol epoxides and subsequent formation of stable DNA adducts is responsible for mutation induction in these cells.

In contrast to the results obtained with DB[*a*,*l*]P, the presence of AP sites was detected in DNA from MCF-7 cells after treatment with DMBA or B[*a*]P (Figure 2). It has been reported that at least 99% of all DNA adducts formed during incubation of isolated DNA with DMBA in the presence of rat liver microsomes or in mouse skin after application of DMBA were unstable and depurinate to form AP sites (*21*, *22*). In the study presented here, the proportion of AP sites in DNA of MCF-7 cells after exposure to DMBA and B[*a*]P for 4 h was about 70% of the total DNA lesions detected for both PAH (Figure 2). However, after this short exposure period, the total amount of DNA damage was very low, approximately eight lesions per 10^6 nucleotides. In contrast, after exposure for 24 h, stable DNA adducts represented more than 85% of all DNA lesions in both DMBA- and B[*a*]P-treated MCF-7 cells, and the total number of lesions had increased more than 5-fold to 38 lesions per 10^6 nucleotides. These results indicate that AP sites in DNA represent a substantial proportion of the total DNA damage only after short periods of exposure. Shortly after treatment, the level of metabolic activation of DMBA and B[*a*]P to diol epoxides is low, presumably due to limited induction of P450 enzymes (*57*, *58*). At later times, stable DNA adducts represent the majority of all DNA lesions induced by DMBA or B[*a*]P.

The absence of induction of AP sites during metabolic activation of carcinogenic PAH to diol epoxides is consistent with our previous findings that exclusively stable adducts were present after exposure of DNA in solution or mammalian cells in culture to DB[*a*,*l*]P or its 11,12-diol 13,14-epoxides (*29*, *38*). Using the corresponding bay region *anti*-7,8-diol 9,10-epoxide of B[*a*]P, King et al. (*59*) reported that only small proportions of DNA adducts were formed by covalent interaction with the N7 position of guanine bases to generate depurinating reaction products, and Drouin et al. (*60*) demonstrated that AP sites were not involved in mutagenicity caused by DNA adducts of this compound. Osborne et al. (*61*) examined the ability of both diastereomeric *syn*- and *anti*-7,8-diol 9,10-epoxides of B[*a*]P to induce alkaline-labile sites in DNA and found that these sites account for less than 2% of total DNA binding. All these results are consistent with recently published evidence that diol epoxides of strong carcinogens such as B[*a*]P induced only a small fraction of labile but a large fraction of stable and persistent DNA adducts in vitro (*62*). In contrast, diol epoxides from weak carcinogens such as chrysene were demonstrated to induce a time-dependent accumulation of AP sites over 24 h and, moreover, during a period of a few weeks at −20 °C (*62*). The absence of biological consequences upon exposure to chrysene and other PAH that induce high numbers of AP sites by depurination of unstable adducts should be due to the very effective repair of these lesions catalyzed by the base excision repair pathway (*62*−*65*). On the other side, stable DNA adducts are repaired by the nucleotide excision repair pathway which is slow and biochemically complex and, therefore, may be prone to producing errors during the repair process (*62*−*64*).

Incubation of DNA in solution with PAH in the presence of horseradish peroxidase and appropriate cofactors was demonstrated to result in the formation of depurinating adducts (*66*−*68*). It has been proposed that formation of depurinating DNA adducts of PAH possessing an ionization potential of less than 7.35 eV occurs via metabolically catalyzed generation of radical cations by a one-electron oxidation step, a condition that would be fulfilled by all three PAH investigated in the study presented here, B[*a*]P (7.23 eV), DMBA (7.22 eV), and DB[*a*,*l*]P (7.26 eV) (*69*, *70*). To investigate whether a high peroxidase activity expressed in human cells could contribute to an increase in the level of AP sites in genomic DNA upon exposure to PAH, human leukemia HL-60 cells were treated with B[*a*]P, DMBA, or DB[*a*,*l*]P. No statistically significant increase in the number of AP sites was obtained with any PAH even at concentrations as high as 30 μM (Figure 5). HL-60 cells also failed to produce stable DNA adducts from any of the three PAH or water-soluble metabolites from [^{3}H]B[*a*]P. Although HL-60 cells are known to contain high peroxidase activity and have been found to catalyze DNA binding of benzene via metabolic formation of hydroquinone (*30*), the results obtained in the study presented here demonstrate that carcinogenic polynuclear aromatic compounds require P450 activity to elicit the formation of high levels of stable DNA adducts as well as the generation of low numbers of AP sites (Figures 2 and 5).

The data presented here indicate that P450-catalyzed activation of the strong carcinogens B[*a*]P, DMBA, and DB[*a*,*l*]P to bay or fjord region diol epoxides, rather than the formation of radical cations via one-electron oxidation, is predominantly responsible for the DNA damage induced by these carcinogenic PAH in mammalian cells. The absence of any significant increase in the number of AP sites, together with the exceptionally high level of stable adducts in cellular DNA after exposure to the most potent carcinogen DB[*a*,*l*]P, leads to the conclusion that formation of stable PAH−DNA adducts rather than the induction of AP sites by depurinating adducts is the

crucial molecular event responsible for the high mutagenic and carcinogenic potency of PAH.

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