Comparison of Cytochrome P450- and Peroxidase-dependent Metabolic Activation of the Potent Carcinogen Dibenzo[a,l]pyrene in Human Cell Lines: Formation of Stable DNA Adducts and Absence of a Detectable Increase in Apurinic Sites

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Abstract

The potent carcinogen dibenzo[a,l]pyrene (DB[a,l]P) has been reported to form both stable and depurinating DNA adducts upon activation by cytochrome P450 enzymes and/or cellular peroxidases. Only stable DB[a,l]P-DNA adducts were detected in DNA after reaction of DB[a,l]P-11,12-diol-13,14-epoxides in solution or cells in culture. To determine whether DB[a,l]P can be activated to metabolites that form depurinating adducts in cells with either high peroxidase (human leukemia HL-60 cell line) or cytochrome P450 activity (human mammary carcinoma MCF-7 cell line), cultures were treated with DB[a,l]P for 4 h, and the levels of stable adducts and apurinic (AP) sites in the DNA were determined. DNA samples from DB[a,l]P-treated HL-60 cells contained no detectable levels of either stable adducts or AP sites. MCF-7 cells exposed to 2 μM DB[a,l]P for 4 h contained 4 stable adducts per 10^8 nucleotides, but no detectable increase in AP sites. The results indicate that metabolic activation of DB[a,l]P by cytochrome P450 enzymes to diol epoxides that form stable DNA adducts, rather than one-electron oxidation catalyzed either by cytochrome P450 enzymes or peroxidases to form AP sites, is responsible for the high carcinogenic activity of DB[a,l]P.

Introduction

Studies of the DNA adducts formed by PAHs have played a key role in elucidating the mechanisms responsible for the metabolic activation of these chemical carcinogens. On the basis of metabolism studies and the structures of the DNA adducts formed, two pathways for the activation of this class of compounds have been described. The monooxygenation route catalyzed by cytochrome P450 enzymes forms bay- or fjord-region diol epoxides via intermediate formation of aren oxide and their hydrolysis products, dihydrodiols. The second activation route, proposed by Cavaliere et al. (2), involves a one-electron oxidation step that generates reactive radical cation intermediates that bind to cellular DNA.

Extensive evidence has been compiled in numerous laboratories (1) that vicinal diol epoxides represent ultimate carcinogenic metabolites of most PAHs. However, it has been demonstrated that radical cation intermediates of PAH can be generated by removal of a π electron; this could be catalyzed by cellular peroxidases and/or, potentially, by cytochrome P450 (2, 3). These intermediates can react with DNA to form predominantly adducts at the N7 position of purine bases, which destabilize the glycosidic bonds and cause spontaneous depurination, resulting in the generation of AP sites (4). It has been proposed that most PAH-DNA binding catalyzed by isolated rat liver nuclei and microsomes results from formation of PAH radical cations (5). Many of the DNA adducts formed by radical cations were found to be unstable, and the failure to detect adducts formed by radical cations in DNA could be due to their loss by depurination (2).

Recent investigations on the metabolic activation of the potent carcinogen DB[a,l]P by rat liver microsomes suggested that both mechanisms were involved but that 84% of all DB[a,l]P-DNA adducts formed were unstable (1). A comparable prevalence of depurinating adducts has also been described for microsome-catalyzed DNA binding of benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene, and the percentages of unstable adducts were 80 and 99% for benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene, respectively (4, 6). Because stable DB[a,l]PDE-DNA adducts represented only a small portion of all DB[a,l]P-DNA adducts detected, these findings would imply that at least two-thirds of all DB[a,l]PDE adducts are unstable (3). It has been hypothesized that AP sites formed by depurination of unstable adducts are involved in tumor initiation by DB[a,l]P (6).

To determine the relative contribution of these two different mechanisms of activation of the potent carcinogen DB[a,l]P in damaging cellular DNA, we previously used an alkaline cleavage-Southern blot assay to determine the proportions of stable adducts and AP sites formed by reaction of DB[a,l]P and DB[a,l]PDEs with DNA in solution and cells in culture (7). No AP sites were detected in either the diol epoxide-treated DNA or the DNA obtained from cells after exposure to DB[a,l]P (7). However, after incubation of DNA with DB[a,l]P and HRP in vitro, detectable levels of AP sites were formed, indicating that unstable adducts were produced (7).

Here, we compared the ability of cellular peroxidase and P450 activity in intact human cells to activate DB[a,l]P to intermediates that damage DNA. To increase the sensitivity of detection of AP sites in cellular DNA, we used a new aldehyde reactive reagent in a sensitive ELISA-type assay (8). The proportions of stable adducts and AP sites formed upon exposure to DB[a,l]P were investigated in: (a) the human promyelocytic leukemia HL-60 cell line with high intracellular peroxidase activity that lacks the P450 enzymes responsible for activation of PAH to diol epoxides (9, 10) and (b) the human mammary carcinoma MCF-7 cell line with both P450 1A1 and P450 1B1 but no detectable peroxidase activity.

Materials and Methods

Chemicals. DB[a,l]P was obtained from Chemsyn Science Laboratories (Lenexa, KS). The NARP reagent, N(+) -biotinyl-l-lysine hydrazide, was ob-
tained from Fluka Chemical Co. (Milwaukee, WI), and the ARP was synthe-
sized as previously described by Kubo et al. (8). Bicinchoninic acid protein
reagents were purchased from Pierce (Rockford, IL). HRP (grade VI, EC
1.11.1.7), guaiacol, and hydrogen peroxide were obtained from Sigma Chem-
ical Co. (St. Louis, MO). An ELISA Amplification System was purchased from
Life Technologies, Inc. (Gaithersburg, MD).

Cell Culture Conditions. The human mammary carcinoma MCF-7 and
the promyelocytic-leukemia HL-60 cells were grown in 175-cm² flasks with
DMEM/F-12 medium (1:1 mixture) supplemented with 10% FCS, 15 mM
HEPES buffer, 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% CO₂/95% air atmosphere.

Peroxidase Activity Assay. Two aliquots of HL-60 cells (10⁷) were resus-
pended and homogenized in lysis buffer [0.25 M sucrose, 20 mM Tris-HCl (pH
7.4), 100 mM KCl, 40 mM NaCl, and 10 mM MgCl₂] for enzyme activity
analysis at 4°C (11). MCF-7 cells were also resuspended in this lysis buffer and
analyzed for intracellular peroxidase activity. After homogenization at 4°C,
the cell lysates were centrifuged at 5000 × g for 10 min at 4°C (9). The
supernatant of one aliquot was saved for analysis of the peroxidase activity,
and the other aliquot was centrifuged at 105,000 × g for 90 min at 4°C. After
this additional centrifugation, that supernatant was saved, and the pellet
was resuspended in microsome dilution buffer [0.1 M KH₂PO₄, 20% glycerol, 10
mM EDTA, 0.1 mM DTT, and 0.25 mM phenylmethylsulfonyl fluoride (pH
7.4)]. Protein concentrations were determined using the bicinchoninic acid
assay (Pierce).

Peroxidase activity was measured using guaiacol as a substrate (12, 13).
The reaction was initiated by adding an aliquot of the isolated protein solution to
0.1 M phosphate buffer (pH 7.4) containing 33 mM guaiacol and 0.27 mM
hydrogen peroxide (final volume, 1 ml). Oxidation of guaiacol was measured
spectrophotometrically at 470 nm and 25°C for 3 min (12). Peroxidase activity
was reported as the increase in absorbance at 470 nm due to the formation of
tetraguaiacol per min and ng of total protein in the reaction mixture. The
oxidation of guaiacol reached a maximum in absorbance at −0.5 min, a finding
also observed by others using this assay for different purified peroxidases (12).
An extinction coefficient of 26.6 mM⁻¹ cm⁻¹ was used to quantitate tetraguai-
acol formation (13).

Cell Treatment and Preparation of DNA. After MCF-7 cells had covered
>90% of the surface area of the 175-cm² flask, the medium was replaced with
50 ml of fresh medium containing 10% serum. Twenty-four h later, cells
were treated with DMSO alone or with 1 or 2 mM DB[a,l]P. The cells were harvested
4 h after treatment, and the DNA was isolated by treatment with RNase,
proteinase K, and phenol, followed by chloroform:isoamyl alcohol (24:1)
extractions, as described previously (7). HL-60 cells were grown in suspension
in 100 ml of medium (9), and −10⁷ cells were treated with 2, 4, or 6 mM
DB[a,l]P for 4 h. The cells were then collected by centrifugation at 3000 × g
for 10 min, and DNA was isolated as described above.

Samples of DNA reacted with DB[a,l]P in the presence of HRP-H₂O₂ were
prepared as described previously (7).

Microtiter Plate Assay for AP Sites. The assay for AP sites was carried out
according to the protocol described previously by Kubo et al. (8), with some
minor modifications. Polystyrene 96-well microtiter plates (Corning,
Corning, NY) were coated with 2 µg of DNA per well dissolved in 100 µl of
TBS buffer [0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl] and incubated uncovered
at 37°C overnight to fix the DNA to the wells. The plates were then washed
four times with 100 µl of TBSB [TBS containing 0.5% Tween 20], and 100 µl
of NARP reagent (1 mM) were added for 1 h at 37°C. The wells were washed
four times with TBSB to remove unreacted NARP reagent. Nonspecific bind-
ing sites were blocked with 100 µl of TBSB per well containing 1% calf serum
by incubation for 1 h at 37°C. The wells were washed four times with TBSB,
and the biotin-tagged AP sites were then detected by adding 50 µl of a streptavidin-alkaline phosphatase complex (1:8000 dilution in TBSB with 1%
calf serum; Life Technologies, Inc.) for 1 h at 37°C. The wells were washed
eight times with TBST to remove unreacted NARP reagent. Nonspecific bind-
ing sites were then detected by adding 50 µl of streptavidin-alkaline phosphatase
complex (1:8000 dilution in TBSB with 1% calf serum; Life Technologies, Inc.) for 1 h at 37°C. The wells were washed eight times with TBSB, and an ELISA amplification system kit was used (as
described by the vendor) to quantitate the color formation at 495 nm using a
Spectra Plate Reader System from Molecular Devices Co. (Sunnyvale, CA).

Methylated DNA obtained from Chinese hamster ovary cells containing
known numbers of AP sites (7) was used for external standardization.

³²P-Postlabeling and HPLC Analysis. Ten µg of DNA obtained from
MCF-7 or HL-60 cells after treatment with DB[a,l]P were ³²P-postlabeled
using the nuclease P1 and prostatic acid phosphatase protocol, as described
previously (7). ³²P-labeled DNA adducts were purified by Sep-Pak C₁₈
column chromatography and subsequently subjected to HPLC analysis using a
5-µm Ultrasphere C₁₈ 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), as described previously (7).

Results and Discussion

To investigate the role of one-electron oxidation catalyzed by
peroxidases in the activation of PAH to reactive intermediates that
form depurinating DNA adducts, two human cell lines were evaluated
for peroxidase activity. Peroxidase activity was measured by
the oxidation of guaiacol (12, 13). HRP was used to establish conditions
for the assay as well as a positive control in all measurements of
acellular fractions (data not shown). Cell pellets of the human leukemia
cell line HL-60 were used to prepare a 5000 × g supernatant as well as
a 105,000 × g microsomal fraction and its corresponding super-
natant. Assays of guaiacol oxidation using 1.4 mg of total protein
from each fraction demonstrated significant peroxidase activity in all
preparations. The 5000 × g supernatant of HL-60 cells catalyzed
oxidative turnover of 94 nmol/min/mg protein. Fractionation of this
supernatant demonstrated that the activity was present in both
the microsomal (81 nmol/min/mg protein) and the 105,000 × g super-
natant fraction (9 nmol/min/mg protein).

The peroxidase activity of MCF-7 cells was analyzed by measure-
ments of guaiacol oxidation for the same cellular fractions described
above. Even at concentrations as high as 5 mg total protein per
sample, no peroxidase activity could be detected either in the
5000 × g supernatant or in microsomes. Previous studies with MCF-7
cell cultures revealed that these cells possess constitutively expressed
P450 1B1 as well as inducible P450 1B1 activity and inducible P450
1A1 activity (14). In contrast, HL-60 cells have previously been shown
to lack inducible P450 1A1 and contain very low levels of aryl
hydrocarbon hydroxylase activity (10). The presence of high peroxi-
dase activity and absence of P450 mediated metabolism of PAH in
HL-60 cells, together with P450 catalyzed metabolic turnover of PAH
and absence of detectable peroxidase activity in MCF-7 cells, allowed
the evaluation of the role of each of these pathways in PAH activation
in human cell cultures.

To measure DNA damage caused by metabolites of PAH that form
depurinating adducts, we previously used an assay for AP sites that
measured the integrity of a restriction fragment of the dihydrofolate
reductase gene after alkaline hydrolysis using Southern blotting tech-
niques (7). This assay is based upon hybridization of a ³²P-labeled riboprobe specific for a sequence in the transcription template strand of
a 20 kb KpnI fragment at the 5’ end of the human dihydrofolate
reductase gene (15). Previous results had demonstrated that the sen-
sitivity of this assay is limited to 1 AP site per 60,000 bases, especially
in cells that contain only single copy of this gene. To ensure a higher
sensitivity for detection of small variations from the basal level of AP
sites present in cells, we used a modification of the ARP assay
described by Kubo et al. (8) in this study.

The ARP assay is based on the reaction of a biotin-tagged ARP
reagent (Fig. 1) with the aldehyde groups formed from deoxyriboses
after release of the purine bases. Quantitation of the biotin-tagged AP
sites can be performed colorimetrically using the avidin/biotin complex
conjugated to either HRP or alkaline phosphatase as the indicator
enzyme (8). The assay was modified to use a commercially available
biotin-tagged reagent that reacts with aldehyde groups formed in
depurinated sugar residues of DNA. The NARP [N-(+)-biotinyl-L-
lysinehydrazide] contains a lysine residue at the amide linkage that
is bound to a hydrazide group (Fig. 1), in contrast to the original
compound used by Kubo et al. (8) which contains an alkoxyamine

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DB[a,l]P forms stable adducts, not AP sites, in DNA

DNA adducts can be detected with high sensitivity by the NARP reagent.

Analysis of DNA obtained from MCF-7 cells after exposure to DB[a,l]P for 24 h by the Southern blot technique failed to detect any increase in AP sites (7). Several reports on the induction of AP sites by formation of unstable DB[a,l]P-DNA adducts used shorter treatment times, such as 4 h (16). To determine whether AP sites were present after a short period of treatment, we exposed MCF-7 and HL-60 cells to various concentrations of DB[a,l]P for 4 h. DNA obtained from MCF-7 cells after exposure to 1 or 2 μM DB[a,l]P for 4 h contained only background levels of AP sites (Fig. 3B). The DNA obtained from HL-60 cells after treatment with DB[a,l]P in a concentration range from 2 to 6 μM for 4 h also contained only background levels of AP sites (Fig. 3C). In both experiments, DNA treated with DMS as a positive control gave high absorbance values. In contrast, the DMS-treated sample gave values close to the background level after addition of methoxyamine (Fig. 3). These results indicate that neither DNA from cells expressing high P450 activity nor DNA from cells expressing high peroxidase activity contained significantly increased numbers of AP sites after exposure of the cells to DB[a,l]P.

The ability of the assay to detect AP sites in DNA after exposure to DB[a,l]P in the presence of HRP was investigated by treatment of DNA obtained from CHO-B11 cells with various concentrations of DB[a,l]P in the presence of HRP and hydrogen peroxide. A positive correlation was found between increasing dose of DB[a,l]P and absorbance at 495 nm. At all doses, a significant increase in AP sites above background was observed (Fig. 3A). DNA samples incubated with methoxyamine to protect the aldehyde group from reacting with the aldehyde-reactive reagents prior to the NARP assay. The microtiter plate wells were then coated with 2 μg of DNA. NARP reagent was added, and the biotin-tagged AP sites were measured using a streptavidin-alkaline phosphatase complex. Columns, means of three independent determinations; bars, SD. * values were significantly different from the control (A) or DMSO samples (B or C; P < 0.05), as determined by the Student’s t test.
Stable DB[a,l]P-DNA adducts formed in MCF-7 and HL-60 cells as well as those formed during incubation with HRP and H$_2$O$_2$ were investigated by $^{33}$P-postlabeling and HPLC analysis. The HPLC elution profiles of adducts in MCF-7 cells after treatment with 1 or 2 $\mu$m DB[a,l]P for 4 h are shown in Fig. 4, A and B, respectively. In both experiments, three major adduct peaks were present which have been previously demonstrated to result from the reaction of DB[a,l]PDEs with DNA (17). The peaks eluting at 71 and 103 min are deoxyadenosine adducts of the (+)-syn-DB[a,l]PDE and the peak eluting at 74 min is a (−)-anti-DB[a,l]PDE-deoxyadenosine adduct (18). The level of adduct formation at 4 h was considerably lower than that previously observed after 24 h of exposure. After treatment with 2 $\mu$m DB[a,l]P for 4 h, ~4 adducts per 10$^6$ nucleotides were formed, whereas >300 adducts per 10$^6$ nucleotides were present after 24 h (7). This difference in adduct levels as well as the much higher proportion of syn-DB[a,l]PDE adducts observed at 4 h compared to 24 h probably results from induction of P450 1A1 and P450 1B1 that activate DB[a,l]P to form stable anti-DB[a,l]PDE-DNA adducts. The identity of this DNA adduct is unknown, but the late elution suggests a relatively nonpolar product as might be expected from a reaction of a radical cation of DB[a,l]P to reactive metabolites capable of forming stable and/or depurinating DNA adducts in human cells. Numerous studies have demonstrated that stable PAH-DNA adducts can lead to mutations through misincorporation of nucleotides or deletions (19). Chakravarti et al. (6) proposed that “transforming” mutations induced in the Ha-ras proto-oncogene in mouse skin papillomas may result from misreplication of AP sites formed by depurinating DNA-adducts generated in excess of the repair capacity of the cells. AP sites are known to be mutagenic lesions (20). However, Loeb (20) has calculated that over 10,000 AP sites are spontaneously formed per cell per day, indicating a high repair capacity for AP sites.

Here, the ability of human cells that express either high P450 or high peroxidase activity to generate stable and depurinating DNA adducts upon metabolic activation of DB[a,l]P has been investigated. The results demonstrate that human cells expressing P450 1A1 and 1B1 metabolically transform DB[a,l]P to DB[a,l]PDEs that form stable DNA adducts. No increase in levels of AP sites was observed at either early (4 h) or late (24 h) times after treatment with DB[a,l]P (7). Human cells containing high peroxidase activity failed to activate DB[a,l]P to metabolites that form stable DNA adducts. No increase in AP sites was observed in HL-60 cells treated with DB[a,l]P, although these lesions could be induced by incubation of DNA with DB[a,l]P in the presence of HRP in solution. No evidence for the formation of an increase in AP sites in DNA was found in MCF-7 cells under conditions that produced high levels of stable DNA adducts. These results indicate that DB[a,l]P is activated to metabolites that form mainly stable adducts with DNA in cells in culture. Although it is possible that AP sites formed by depurinating adducts are rapidly repaired in cells and, thus, may not be above background levels, this would suggest that AP sites caused by depurinating DB[a,l]P-DNA adducts are unlikely to contribute significantly to the high mutagenic potency of DB[a,l]P. In conclusion, cytochrome P450-catalyzed activation to DB[a,l]PDEs, rather than cytochrome P450- or peroxidase-mediated one-electron oxidation to radical cations, is responsible for the DNA damage induced by DB[a,l]P in mammalian cells and accounts for the exceptionally high carcinogenic potency of DB[a,l]P.

Acknowledgments

We are grateful to Dr. Marietta Harrison (Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University) and Dr. David Barnes (Department of Biochemistry and Biophysics, Oregon State University) for supplying the human promyelocytic leukemia HL-60 cells.

References


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