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The level of DNA modification by (+)-*syn*-(11*S*,12*R*,13*S*,14*R*)- and (–)-*anti*-(11*R*,12*S*,13*S*,14*R*)-dihydrodiol epoxides of dibenzo[*a*,*l*]pyrene determined the effect on the proteins p53 and p21^{WAF1} in the human mammary carcinoma cell line MCF-7

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The polycyclic aromatic hydrocarbon (PAH) dibenzo[*a*,*l*]-pyrene (DB[*a*,*l*]P), the most carcinogenic PAH tested in rodent bioassays, exerts its pathobiological activity via metabolic formation of electrophilically reactive DNA-binding fjord region (+)-*syn*-(11*S*,12*R*,13*S*,14*R*)- or (–)-*anti*-(11*R*,12*S*,13*S*,14*R*)-DB[*a*,*l*]P-dihydrodiol epoxides (DB[*a*,*l*]-PDEs). DB[*a*,*l*]P is metabolized to these DB[*a*,*l*]PDEs which bind to DNA in human mammary carcinoma MCF-7 cells. The molecular response of MCF-7 cells to DNA damage caused by DB[*a*,*l*]PDEs was investigated by analyzing effects on the expression of the tumor suppressor protein p53 and one of its target gene products, the cyclin-dependent kinase inhibitor p21^{WAF1}. Treatment of MCF-7 cells with (+)-*syn*- and (–)-*anti*-DB[*a*,*l*]PDE at a concentration range of 0.001–0.1 μM resulted in DB[*a*,*l*]PDE–DNA adduct levels between 2 and 30, and 3 and 80 pmol/mg DNA, respectively, 8 h after exposure. (–)-*anti*-DB[*a*,*l*]PDE exhibited a higher binding efficiency that correlated with a significantly stronger p53 response at low concentrations of the dihydrodiol epoxides. The level of p53 increased by 6–8 h after treatment. The p21^{WAF1} protein amount exceeded control levels by 12 h and remained elevated for 96 h. At a dose of 0.01 μM (+)-*syn*-DB[*a*,*l*]PDE, an increase in p21^{WAF1} was observed in the absence of a detectable change in p53 levels. The results indicate that the increase in p53 induced by DB[*a*,*l*]PDEs in MCF-7 cells requires an adduct level of ~15 pmol/mg DNA and suggest that the level of adducts rather than the specific structure of the DB[*a*,*l*]PDE–DNA adduct formed triggers the p53 response. The PAH–DNA adduct level formed may determine whether p53 and p21^{WAF1} pathways respond, resulting in cell-cycle arrest, or fail to respond and increase the risk of mutation induction by these DNA lesions.

Abbreviations: (–)-*anti*-DB[*a*,*l*]PDE, dibenzo[*a*,*l*]pyrene-11*R*,12*S*-dihydrodiol 13*S*,14*R*-epoxide; (+)-*anti*-B[*a*]PDE, B[*a*]P-7*R*,8*S*-dihydrodiol 9*S*,10*R*-epoxide; (+)-*syn*-DB[*a*,*l*]PDE, dibenzo[*a*,*l*]pyrene-11*S*,12*R*-dihydrodiol 13*S*,14*R*-epoxide; B[*a*]P, benzo[*a*]pyrene; CDK, cyclin-dependent kinase; DB[*a*,*l*]P, dibenzo[*a*,*l*]pyrene; DB[*a*,*l*]PDE(s), DB[*a*,*l*]P-11,12-dihydrodiol 13,14-epoxide(s); DMSO, dimethyl sulfoxide; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; PAH(s), polycyclic aromatic hydrocarbon(s); PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TCPI, trypsin–chymotrypsin protease inhibitor.

Introduction

The hexacyclic aromatic hydrocarbon dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P) (Figure 1) is the most highly tumorigenic polycyclic aromatic hydrocarbon (PAH) tested to date. The tumor-initiating activity of DB[*a*,*l*]P exceeds that of benzo[*a*]pyrene (B[*a*]P) and even of 7,12-dimethylbenz[*a*]anthracene (DMBA), formerly thought to be the most potent carcinogenic PAH after application to mouse skin or rat mammary gland (1–3). DB[*a*,*l*]P has been detected as a widespread pollutant in the human environment (4–8), and several laboratories have investigated the mechanism of DNA damage induction by this compound. Studies in mammalian cell cultures including human cell lines (9,10), in mouse skin *in vivo* (9), and in microsomal preparations (11) revealed that cytochrome P450 enzymes activate DB[*a*,*l*]P to its electrophilically reactive fjord region 11,12-dihydrodiol 13,14-epoxides (DB[*a*,*l*]PDEs) (Figure 1) which predominantly bind to deoxyadenosine residues within DNA. Analysis of the stereochemical course of the metabolic activation in human mammary carcinoma MCF-7 cells (10) demonstrated that this PAH is exclusively converted to (+)-*syn*-(11*S*,12*R*,13*S*,14*R*)- and (–)-*anti*-(11*R*,12*S*,13*S*,14*R*)-DB[*a*,*l*]PDE via their corresponding precursors, the (+)-(11*S*,12*S*)- and (–)-(11*R*,12*R*)-dihydrodiols, respectively (Figure 1). No formation of (+)-*anti*-(11*S*,12*R*,13*R*,14*S*)- and (–)-*syn*-(11*R*,12*S*,13*R*,14*S*)-DB[*a*,*l*]PDE was detected (10). Although only racemic fjord region *syn*- and *anti*-DB[*a*,*l*]PDEs have been tested, their extraordinarily strong mutagenic activity in *Salmonella typhimurium* and Chinese hamster V79 cells (12), and their high carcinogenic potency in mouse skin, newborn mouse and rat mammary gland (13–15) may account for the high tumorigenicity of DB[*a*,*l*]P. Although it has been proposed that DB[*a*,*l*]P can also be activated through a radical cation intermediate to produce unstable depurinating DNA adducts (11), no increase in apurinic sites was detected in MCF-7 cells exposed to DB[*a*,*l*]P and its 11,12-dihydrodiol 13,14-epoxides (16). The DNA damage induced in MCF-7 cells treated with DB[*a*,*l*]P results from the formation of stable covalent DB[*a*,*l*]PDE–DNA adducts only (16).

Covalent modification of genomic DNA by metabolically formed DB[*a*,*l*]PDEs (10,11,17,18) represents a type of cellular DNA damage demonstrated previously to be responsible for an increase in the tumor suppressor protein p53. Cells containing wild-type p53 phosphoprotein are able to recognize DNA damage caused not only by metabolites of PAH (19–22), but also by UV light (23), ionizing radiation (24) or antitumor drugs (25). The initial cellular response consists of a nuclear accumulation of p53, transcriptional induction of various target genes containing p53-binding domains, and subsequent cell-cycle arrest, usually in G_1 (24,26). Within this signal cascade the cyclin-dependent kinase (CDK) inhibitor

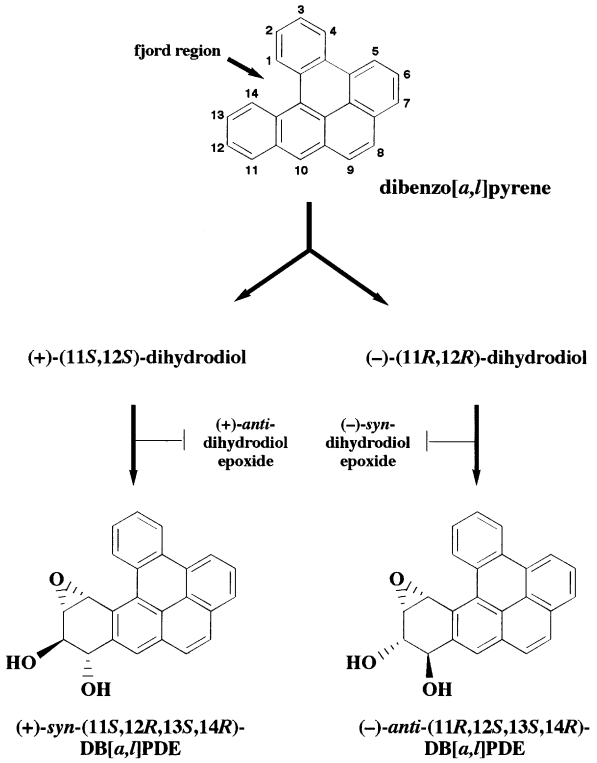

Fig. 1. Schematic representation of the stereoselective metabolism of dibenzo[*a,l*]pyrene in human mammary carcinoma MCF-7 cells.

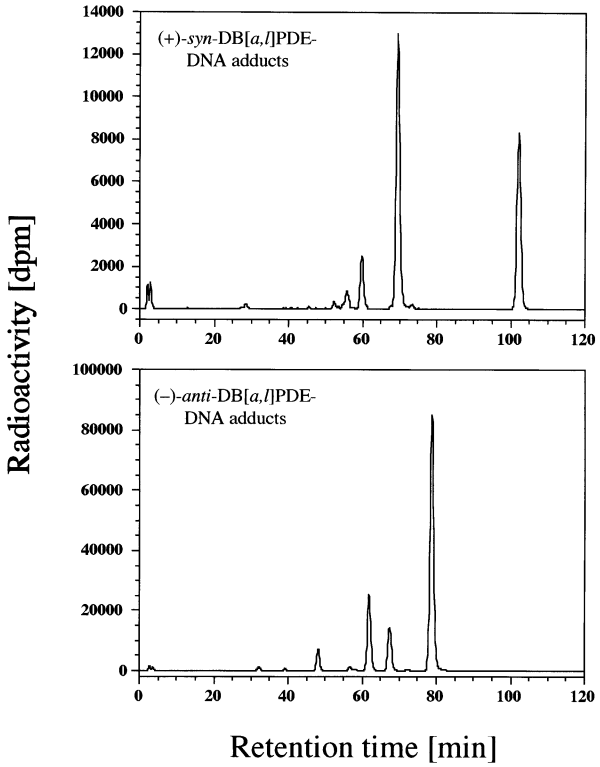

Fig. 2. HPLC elution profiles of ^{33}P-labeled DB[*a,l*]PDE–DNA adducts obtained from DNA of MCF-7 cells exposed to 0.03 μM (+)-*syn*- or (–)-*anti*-DB[*a,l*]PDE. Treatment with (+)-*syn*-DB[*a,l*]PDE resulted in two major DNA adducts that eluted at 70 and 102 min (dA adducts), whereas (–)-*anti*-DB[*a,l*]PDE formed one predominant DNA adduct that eluted at 78 min (dA adduct) and two minor adducts at 62 and 68 min (dG adducts). All adducts were identified by cochromatography with synthetic standards as previously described (10). ^{33}P-post-labeling and separation on HPLC was performed as described in Materials and methods.

p21^{WAF1} is an important mediator of the p53-induced cell-cycle arrest (26,27). Evidence that p53 is involved in DNA repair (28,29) and the induction of apoptosis (26,30) led to recognition that p53 participates in a signal transduction pathway which recognizes DNA damage and which can subsequently lead to growth arrest until DNA damage is repaired or programed cell death has been initiated (31). In contrast, cells which contain a mutated *p53* gene, even if they express high constitutive levels of its protein product, lack a comparable response to DNA damage (21,24) and are more suceptible to the induction of mutations and development of transformed cell clones. The high prevalence of *p53* gene mutations found in human cancers is consistent with this role for the p53 protein (32).

In order to determine how specific PAH–DNA adduct levels determine the cellular response to PAH–DNA damage, wild-type p53-expressing mammary adenocarcinoma-derived MCF-7 cells (33) were treated with (+)-*syn*- and (–)-*anti*-DB[*a,l*]PDE and levels of DNA adducts in conjunction with those of the tumor suppressor protein p53 and the CDK inhibitor p21^{WAF1} were measured over a 96 h period after exposure.

Materials and methods

Chemicals

Nuclease P1 (EC 3.1.30.1; from *Penicillium citrinum*), human prostatic acid phosphatase (EC 3.1.3.2; from human semen), apyrase (EC 3.6.1.5; from *Solanum tuberosum*), phosphodiesterase I (EC 3.1.4.1; from *Crotalus atrox*) and proteinase K (EC 3.4.21.64; from *Tritirachium album*) were purchased from Sigma (St. Louis, MO). RNase T1 (EC 3.1.21.3; from *Aspergillus oryzae*) and RNase (DNase free, a heterogeneous mixture of ribonucleases from bovine pancreas) were obtained from Boehringer Mannheim (Indianapolis, IN). Unequilibrated phenol and cloned T4 polynucleotide kinase were purchased from United States Biochemical (Cleveland, OH). [γ-^{33}P]ATP [3500 Ci (129.5 TBq)/mmol] was purchased from Amersham (Arlington Heights, IL). The protease inhibitors leupeptin, phenylmethylsulfonyl fluoride (PMSF), aprotinin, and the trypsin–chymotrypsin protease inhibitor (TCPI) were obtained from Boehringer Mannheim. Phosphate-buffered saline (PBS) contained 3.0 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, 8.0 mM Na_2HPO_4 (pH 7.4). Acrylamide and bisacrylamide for gel electrophoresis were purchased as a 40% mixture (w/v) from Bio-Rad (Hercules, CA). Preparation of enantiomeric 11,12-dihydrodiols of DB[*a,l*]P as described previously (17) allowed subsequent generation of optically pure (+)-*syn*- and (–)-*anti*-DB[*a,l*]PDEs using the same synthetic route described for the racemic compounds (12).

Cell culture

The human mammary carcinoma cell line MCF-7 (original stock line was obtained from the Michigan Cancer Foundation) was grown in 175 cm^2 cell culture flasks in a total volume of 50 ml of Dulbecco's modified Eagle's medium, high glucose type (DMEM with 4.5 g D-glucose/l; Gibco BRL, Grand Island, NY), supplemented with 10% fetal calf serum (FCS; Intergen, Purchase, NY), 0.1 mM non-essential amino acids (Gibco BRL) and 1 mM sodium pyruvate (Gibco BRL).

Treatment of MCF-7 cells with (+)-syn- and (–)-anti-DB[a,l]PDE

After MCF-7 cells covered ~50–60% of the surface area of the flasks (2–3 days after splitting of a confluent culture), the media was removed and the cells were washed twice with 20 ml sterile PBS. Medium without FCS (50 ml) was added to the flask, then 30 μl of a dimethyl sulfoxide (DMSO) solution of the enantiomerically pure (+)-*syn*- or (–)-*anti*-DB[*a,l*]PDE was added. (Stock solutions of 1 mg/ml DB[*a,l*]PDE were diluted in DMSO to adjust the required concentration.) The cells were treated with the compounds in a concentration range between 0.001 and 0.1 μM. The control groups were treated with 30 μl DMSO alone. After 1 h of exposure, the medium was removed and replaced by medium containing 10% FCS. The cells were harvested at 2, 4, 6, 8, 12, 24, 48, 72 and 96 h after treatment by trypsinization with 0.05% trypsin–EDTA (0.05% trypsin, 0.14 M NaCl, 3 mM KCl, 0.1 M Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM EDTA). After addition of

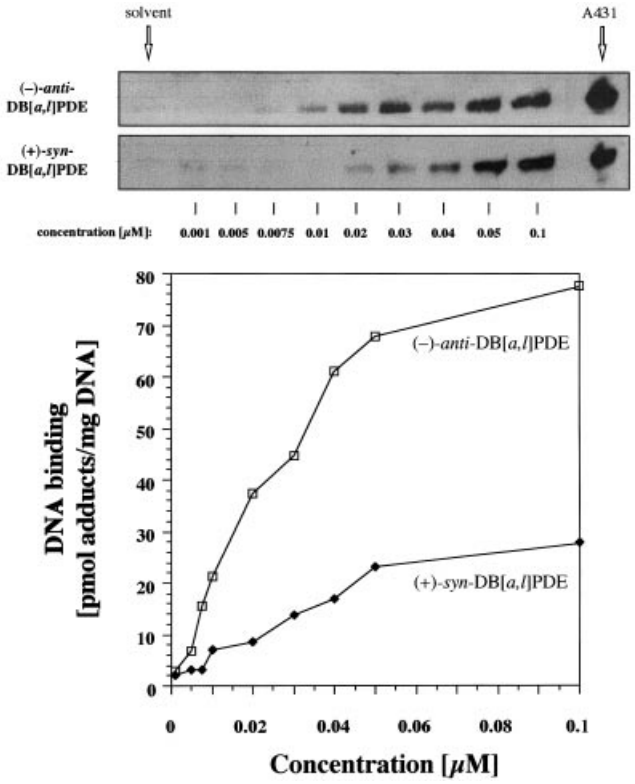

Fig. 3. DNA adduct and p53 protein levels in MCF-7 cells 8 h after exposure to (+)-*syn*- or (–)-*anti*-DB[*a,l*]PDE. Detection of p53 by western blotting and analysis of total DNA binding by post-labeling was performed as described in Materials and methods. A431 (human squamous cell line) protein was included on the blot as a positive control for p53 protein (36).

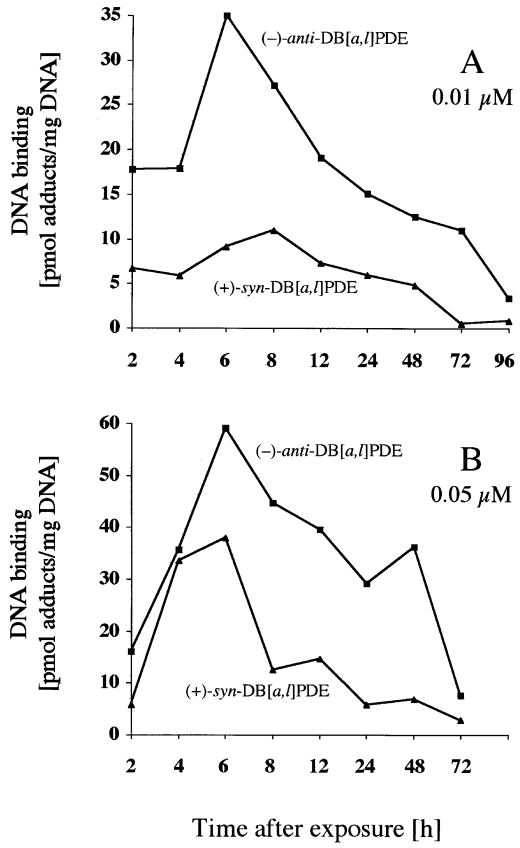

Fig. 4. Total DB[*a,l*]PDE-DNA binding in MCF-7 cells after exposure to (**A**) 0.01 μM and (**B**) 0.05 μM (+)-*syn*- or (–)-*anti*-DB[*a,l*]PDE for the times indicated. Analysis of total DNA binding by post-labeling was performed as described in Materials and methods. Values represent the means of two independent experiments. Individual values varied from the mean within a range of ±35%.

an equal volume of medium containing 10% FCS, the cells were centrifuged at 1000 *g*, washed twice with PBS, and the cell pellet stored at –80°C.

DNA preparation

DNA isolation from MCF-7 cell pellets was carried out as described previously (10). Briefly, the cell pellets were homogenized in EDTA–sodium dodecyl sulfate (SDS) buffer [10 mM Tris, 1 mM Na_2EDTA, 1% SDS (w/v), pH 8] and incubated for 1 h at 37°C with RNase T1 (1000 U/ml) and RNase (DNase free; 5 μg/ml) on a shaker (100 r.p.m.). Then proteinase K (500 μg/ml) was added and the incubation continued for 1 h at 37°C. The mixture was extracted twice with 1 vol Tris-saturated phenol (1 M, pH 8.0) then twice with Tris-saturated phenol/chloroform/*iso*-amyl alcohol (25:24:1, v/v/v). The DNA was precipitated with 2 vol ethanol and 0.1 vol 5 M NaCl, washed with 70% ethanol, dried and dissolved in water. The DNA concentration in the solution was determined by $A_{260\ nm}$.

^{33}P-post-labeling of DB[a,l]PDE-DNA adducts

Post-labeling was carried out as described previously (10). An aliquot of 10 μg DB[*a,l*]PDE–DNA was digested, post-labeled with [γ-^{33}P]ATP (3500 Ci/mmol) and pre-purified with a Sep-Pak C_{18} cartridge (Waters, Milford, MA). Adducts were separated by HPLC on a C_{18} reverse-phase column (5 μm Ultrasphere ODS, 4.6×250 mm; Beckman Instruments) and the radiolabeled nucleotides measured with an on-line radioisotope flow-detector (Radiomatic FLO-ONE Beta; Packard Instruments, Downers Grove, IL). The level of DNA binding (reported as pmol adducts/mg DNA) was calculated based upon the efficiency of labeling of a B[*a*]P-7,8-dihydrodiol 9,10-epoxide–DNA standard as described previously (34).

Isolation and western blotting of MCF-7 cell protein preparations

Total proteins from MCF-7 cells treated with (+)-*syn*- or (–)-*anti*-DB[*a,l*]PDE were isolated according to the protocol described by Harlow and Lane (35). Briefly, the frozen cell pellet was diluted in an appropriate volume of RIPA lysis buffer [150 mM NaCl, 10 mM Tris–HCl (pH 7.2), 1% sodium desoxycholate (w/v), 1% Triton X-100 (v/v), 0.1% SDS (w/v)] (~1 ml/10^7 cells). Prior to addition to the cells the RIPA buffer was pre-chilled to 4°C and the following were added per ml solution: 50 μl of 0.1 M Na_2EDTA (pH 8.0) and 10 μl of each of the protease inhibitors leupeptin (1 mg/ml stock in water), PMSF (100 mM stock in *iso*-propanol), aprotinin (1 mg/ml stock in PBS) and TCPI (1 mg/ml stock in PBS). The buffer–cell mixture was aspirated through a fine-gauge needle (25 gauge fixed on a 1 ml syringe), then boiled in a water bath until an aggregate of sheared DNA and cell fragments had formed (~10 min). After cooling on ice, soluble proteins were separated by centrifugation at 10 000 *g* for 10 min. The protein concentration in this solution was spectrophotometrically determined at 562 nm using the Bicinchoninic acid colorimetric assay of Pierce (Rockford, IL). Lysates of A431 cells (human squamous carcinoma cell line) obtained from the Purdue University cell culture laboratory were also prepared for use as a p53-positive control in western blot analysis (36). These cells contain high amounts of p53 protein due to mutations in codons 248 and 273 of the corresponding gene which result in increased stability of the protein (37).

Prior to the western blotting, an appropriate amount of each isolated protein sample (40 μg) was diluted in loading buffer [10% glycerol (v/v), 5% 2-mercaptoethanol (v/v), 0.16 M Tris (pH 6.8), 3% SDS (w/v), 0.06% bromophenol blue (w/v)] and separated by SDS–polyacrylamide gel electrophoresis (PAGE) (0.1% SDS, 10% acrylamide) using the following electrode buffer: 1.44% glycine (w/v), 0.3% Tris base (w/v), 0.1% SDS (w/v), pH 8.3. After SDS–PAGE, the proteins were transferred onto a nitrocellulose membrane (Bio-Rad) using a transfer buffer consisting of 1.44% glycine (w/v), 0.3% Tris base (w/v), 20% methanol (pH 8.3), then blocked at room temperature with Tris-buffered saline (TBS)–Tween [150 mM NaCl, 0.01 M Tris (pH 8), 0.05% Tween-20 (v/v)] supplemented with 5% (w/v) non-fat dry milk powder for 10 min. After the blocking step had been repeated, the blot was incubated at room temperature for 1 h with the primary antibody diluted in TBS–Tween with 0.5% (w/v) non-fat dry milk powder. For p53 detection, the membrane was incubated with monoclonal antibody p53 Ab-2 (clone Ab 1801; Oncogene Science, Uniondale, NY) which recognizes both the human wild-type and mutant protein. The concentration used was 0.75 μg antibody/ml solution. Monoclonal antibody WAF 1 Ab-1 (clone EA10; Oncogene Science; 0.25 μg antibody/ml solution) was used for measuring the $p21^{WAF1}$ protein. After

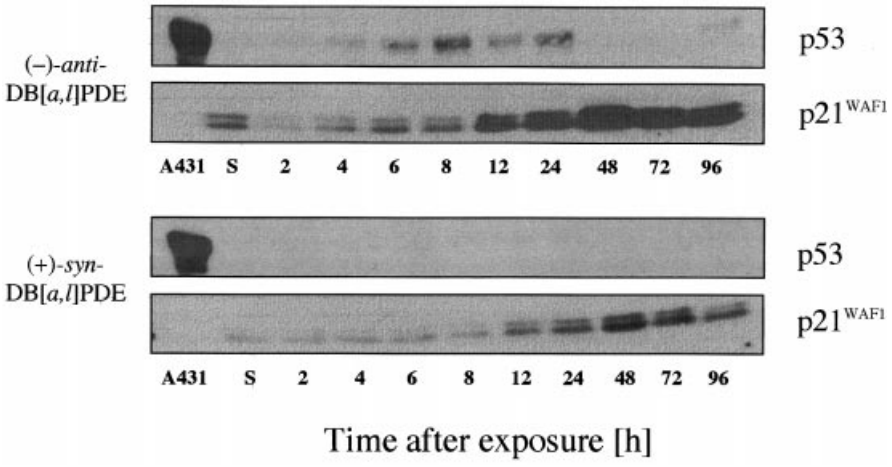

Fig. 5. p53 and p21^{WAF1} protein levels in MCF-7 cells exposed to 0.01 μM (+)-*syn*- or (–)-*anti*-DB[*a,l*]PDE. At the times indicated treatment, harvesting, protein isolation and detection of p53 and p21^{WAF1} were performed as described in Material and methods. A431 (human squamous cell line) protein was included on the blot as a positive control for p53 protein (36). S, solvent (DMSO)-treated control cells.

incubation with the primary antibody the blot was washed twice with TBS–Tween for 10 min each and then incubated for 1 h at room temperature with the secondary antibody (goat anti-mouse IgG linked to horseradish peroxidase) diluted in TBS–Tween. The membranes were washed three times with TBS–Tween and the proteins were detected using the enhanced chemiluminescence technique (Amersham). Protein lysates from A431 cells were used as a positive control for p53.

Results

Human mammary carcinoma MCF-7 cells were treated with enantomerically pure (+)-*syn*- or (–)-*anti*-DB[*a,l*]PDE (Figure 1) in order to determine how DNA adduct formation affected the cellular content of the tumor suppressor p53 protein and the CDK-inhibitor p21^{WAF1}. The HPLC elution profiles of the ^{33}P-post-labeled DNA adducts formed in MCF-7 cells after treatment with 0.03 μM (+)-*syn*- and (–)-*anti*-DB[*a,l*]PDE are shown in Figure 2. The DNA from MCF-7 cells treated with (+)-*syn*-DB[*a,l*]PDE contained two major adduct peaks that eluted at 70 and 102 min. These have previously been identified as dA adducts by cochromatography with synthetic standards (10). The DNA from MCF-7 cells treated with (–)-*anti*-DB[*a,l*]PDE contained three major adduct peaks. The large peak eluted at 78 min is a dA adduct and the smaller peaks eluted at 62 and 68 min are dG adducts (10). At all doses of these dihydrodiol epoxides tested, the proportions of the DNA adducts present were similar; however, the absolute amounts of DNA adducts varied with the dose of dihydrodiol epoxides.

The level of DNA binding and amount of p53 protein in MCF-7 cells observed 8 h after treatment with increasing concentrations of (+)-*syn*- and (–)-*anti*-DB[*a,l*]PDE are shown in Figure 3. For both dihydrodiol epoxides the amount of DNA adducts present increased with dose. Exposure to (–)-*anti*-DB[*a,l*]PDE resulted in 3- to 4-fold higher DNA adduct formation than treatment with the same dose of (+)-*syn*-DB[*a,l*]PDE. An increase in p53 protein levels was also detected at low concentrations of (–)-*anti*-DB[*a,l*]PDE. Whereas treatment with 0.01 μM (–)-*anti*-DB[*a,l*]PDE caused a detectable increase in p53, the threshold dose for a visible increase in p53 was in a range between 0.02 and 0.03 μM in cells treated with the diastereomeric (+)-*syn*-DB[*a,l*]PDE (Figure 3). The adduct level at which an increase in p53 levels was observed was in a comparable range of ~15–20 pmol/mg DNA for both dihydrodiol epoxides.

Measurement of DNA adduct levels at different times after exposure to 0.01 and 0.05 μM (+)-*syn*- or (–)-*anti*-DB[*a,l*]PDE also demonstrated the considerably greater amount of reaction of the (–)-*anti*-diastereomer with DNA in these cells (Figure 4). The maximal DNA adduct levels after treatment with 0.05 μM (+)-*syn*-DB[*a,l*]PDE or 0.01 μM (–)-*anti*-DB[*a,l*]PDE were comparable (38 versus 35 pmol adducts/mg DNA). Almost 60 pmol adducts/mg DNA were obtained 6 h after incubation of MCF-7 cells with 0.05 μM (–)-*anti*-DB[*a,l*]PDE, a modification level of ~1 adduct/50 000 nucleotides. DNA adduction reached maximal levels 6–8 h after exposure for both of the DB[*a,l*]PDE diastereomers used (Figure 4). The amount of adducts subsequently decreased and reached 0.8 and 3.4 pmol/mg DNA 96 h after exposure to 0.01 μM (+)-*syn*- or (–)-*anti*-DB[*a,l*]PDE (Figure 4).

Western blot analysis of p53 and p21^{WAF1} protein levels in MCF-7 cells after incubation with 0.01 μM (+)-*syn*- or (–)-*anti*-DB[*a,l*]PDE are shown in Figure 5. Levels of p53 exceeded control values between 4 and 6 h and reached the maximum level by 8 h after treatment with 0.01 μM (–)-*anti*-DB[*a,l*]PDE. Subsequently, the amount decreased and returned to control values after 48 h. A large increase in p21^{WAF1} was observed ~8 h after treatment with (–)-*anti*-DB[*a,l*]PDE (Figure 5). The level of this protein remained elevated at all times examined up to 96 h. In contrast, no visible accumulation of the p53 protein was detected at any time after treatment of MCF-7 cells with 0.01 μM (+)-*syn*-DB[*a,l*]PDE (Figure 5). Although treatment with 0.01 μM (+)-*syn*-DB[*a,l*]PDE did not cause a detectable increase in p53 protein, this treatment did result in a considerable increase in the amount of the p21^{WAF1} protein (Figure 5). This increase was detectable after 12 h of exposure and persisted through 96 h.

To determine whether an intermediate dose of (+)-*syn*-DB[*a,l*]PDE that gave DNA binding levels comparable with that of 0.01 μM (–)-*anti*-DB[*a,l*]PDE (Figure 3) would result in similar effects on p53 and p21^{WAF1} levels, cells were treated with 0.025 μM (+)-*syn*-DB[*a,l*]PDE for up to 96 h. The western blots shown in Figure 6 demonstrate that this dose of (+)-*syn*-DB[*a,l*]PDE caused an increase in p53 at 6–8 h and a detectable increase in p21^{WAF1} at 12 h that persisted throughout 96 h. Thus, doses of (+)-*syn*- and (–)-*anti*-DB[*a,l*]PDE

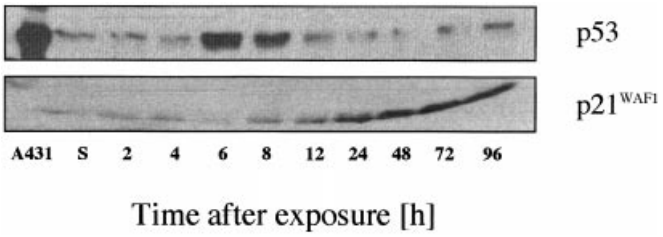

Fig. 6. p53 and p21^{WAF1} protein levels in MCF-7 cells exposed to 0.025 μM (+)-*syn*-DB[*a,l*]PDE. At the times indicated treatment, harvesting, protein isolation and detection of p53 and p21^{WAF1} were performed as described in Material and methods. A431 (human squamous cell line) protein was included on the blot as a positive control for p53 protein (36). S, solvent (DMSO)-treated control cells.

that gave at least 15 pmol adducts/mg DNA caused similar increases in p53 and p21^{WAF1} protein levels.

Discussion

The strong carcinogen DB[*a,l*]P has been found to exert its genotoxic activity in human mammary MCF-7 cells predominantly via metabolic activation to (+)-*syn*- and (–)-*anti*-DB[*a,l*]PDE which react with genomic DNA to form mainly deoxyadenosine adducts (10,16). Measurement of DNA adducts formed in MCF-7 cells after direct incubation with each diastereomeric DB[*a,l*]PDE revealed that (–)-*anti*-DB[*a,l*]PDE caused a 3- to 4-fold higher DNA modification level compared with (+)-*syn*-DB[*a,l*]PDE over a dose range from 0.005 to 0.1 μM (Figure 3). Higher levels of (–)-*anti*-DB[*a,l*]PDE–DNA adducts compared with (+)-*syn*-DB[*a,l*]PDE–DNA adducts were observed in cultures treated with 0.01 or 0.05 μM (–)-*anti*- or (+)-*syn*-DB[*a,l*]PDE over the period of 2–96 h after exposure (Figure 4). The significantly lower DNA adduct level observed after exposure to equimolar concentrations of (+)-*syn*-DB[*a,l*]PDE compared with (–)-*anti*-DB[*a,l*]PDE might be due to increased sequestration of the *syn*-diastereomer as a consequence of its preferentially adopted aligned conformation (12). Vicinal *syn*-dihydrodiol epoxides preferring this conformation have been shown to undergo significantly accelerated solvolytic opening of their oxiranyl ring under neutral conditions compared with corresponding *anti*-diastereomers (38). This explanation would also be consistent with the observation that (+)-*syn*-DB[*a,l*]PDE–DNA adducts were only detected in MCF-7 cells after treatment with high doses of the parent PAH (1–8 μM), whereas (–)-*anti*-DB[*a,l*]PDE–DNA adducts were present at detectable levels after exposure to a dose as low as 0.005 μM DB[*a,l*]P (39).

DNA damage induced by both DB[*a,l*]PDEs increased the cellular content of the p53 protein 8 h after exposure (Figure 3). Therefore, DB[*a,l*]PDE–DNA adducts caused a similar increase in p53 protein levels in MCF-7 cells as has been observed in various human cell cultures treated with a number of DNA-damaging agents including ionizing radiation (24), antitumor drugs (25) and metabolites of other PAHs (19,21,22). A dose as low as 0.01 μM (–)-*anti*-DB[*a,l*]PDE caused a detectable increase in the level of the p53 protein, but 0.02–0.03 μM (+)-*syn*-DB[*a,l*]PDE was required to cause a comparable p53 increase (Figure 3). Based upon the respective level of DNA adducts formed by these dihydrodiol epoxides (Figure 3), (+)-*syn*-DB[*a,l*]PDE-DNA adducts were essentially as effective per adduct in causing an increase in p53 as (–)-*anti*-DB[*a,l*]PDE-DNA adducts. Compared with the results obtained in a previous study using the ultimate genotoxic metabolite of B[*a*]P, B[*a*]P-7*R*,8*S*-dihydrodiol 9*S*,10*R*-epoxide {(+)-*anti*-B[*a*]PDE} (19), both fjord region DB[*a,l*]PDEs caused significantly higher DNA adduct and p53 protein levels/μM dihydrodiol epoxide. Exposure of MCF-7 cells to 0.03 μM (–)-*anti*-DB[*a,l*]PDE (Figure 3) or 0.3 μM (+)-*anti*-B[*a*]PDE (19), both stereoisomers with *R,S,S,R*-configuration, resulted in a comparable DNA binding (~50 pmol adducts/mg DNA) and increases in p53 protein levels. These findings indicate that irrespective of the structure of the specific PAH–DNA adduct formed, doses of (+)-*anti*-B[*a*]PDE, (–)-*anti*-DB[*a,l*]PDE or (+)-*syn*-DB[*a,l*]PDE that formed the same levels of adducts resulted in similar increases in p53 protein levels.

The effect of the DB[*a,l*]PDEs on cellular levels of p53 protein and p21^{WAF1} protein were similar at various times for doses that gave similar DNA adduct levels. Levels of p53 in MCF-7 cells increased to a detectable extent by 4–6 h after exposure to 0.01 μM (–)-*anti*-DB[*a,l*]PDE (Figure 5) or 0.025 μM (+)-*syn*-DB[*a,l*]PDE (Figure 6). In both cultures a large increase in p21^{WAF1} protein was observed after 8–10 h and p21^{WAF1} levels remained elevated up to 96 h (Figures 5 and 6). The time lag observed between p53 response and induction of p21^{WAF1} after treatment with 0.01 μM (–)-*anti*-DB[*a,l*]PDE or 0.025 μM (+)-*syn*-DB[*a,l*]PDE is consistent with a temporal connection between these increases. Others have demonstrated in various wild-type p53-expressing human cell lines that DNA damage leads to nuclear accumulation of this protein followed by induction of p21^{WAF1} and subsequent cell-cycle arrest in G_1 (24,26,27,40). In addition to this p53-dependent signal transduction pathway via induction of p21^{WAF1}, evidence has been found for p53-independent induction of p21^{WAF1} caused by DNA damage (41). The results obtained after treatment of MCF-7 cells with 0.01 μM (+)-*syn*-DB[*a,l*]PDE (Figure 5) may involve such a pathway. Although no increase in p53 was observed at any time after treatment up to 96 h, a considerable increase in p21^{WAF1} protein was detected by 12 h after exposure and maintained for 4 days.

The CDK-inhibitor p21^{WAF1} inhibits both the cyclin-dependent G_1 kinases and the G_2/M-specific cdc2 kinase (42,43). A number of types of DNA damage have been demonstrated to cause G_1 arrest controlled by a wild-type p53-dependent induction of p21^{WAF1} (21,24,44). However, DNA damage can also result in an arrest in G_2. DNA damage induced by γ-irradiation has been found to induce G_2/M accumulation of cells that lack wild-type p53 expression (24,45). Up-regulation of wild-type *p53* gene expression in human fibroblasts in the absence of any DNA damaging agent has also been shown to result in the mediation of a reversible growth arrest by both the control of the G_1 and the G_2/M checkpoints (45). In both cases, the arrest was associated with high levels of p21^{WAF1} (45).

Measurement of the p21^{WAF1} content in MCF-7 cells after treatment with 0.005 μM DB[*a,l*]P (39) or different doses of (–)-*anti*- and (+)-*syn*-DB[*a,l*]PDE (Figures 5 and 6) revealed that the level of this protein did not exceed control values until ~48 h after exposure to the parent PAH or 12–24 h after exposure to both fjord region DB[*a,l*]PDEs. Therefore, any cell-cycle arrest caused by increases in p21^{WAF1} in response to the DB[*a,l*]PDE-induced DNA damage may occur after replication of DNA containing appreciable levels of DB[*a,l*]PDE–DNA adducts. The concept that PAHs can act as

'stealth carcinogens' by allowing replication prior to cell-cycle arrest has been proposed by Khan *et al*. (22). Although they observed cell-cycle arrest in S phase with only a small increase in $p21^{WAF1}$ protein levels in cells treated with racemic *anti*-11,12-dihydrodiol 13,14-epoxide of benzo[*g*]chrysene, the 21 h time-point tested may have been early in the $p21^{WAF1}$ response (22). In studies with DB[*a*,*l*]P (39), B[*a*]P and (+)-*anti*-B[*a*]PDE (19; L.C.Kaspin and W.M.Baird, unpublished results) we observed a cell-cycle arrest in G_2/M in MCF-7 cells and a large increase in $p21^{WAF1}$ levels. The observed cell-cycle arrest in phases other than G_1 may be due to a DNA damage-induced long-term expression of $p21^{WAF1}$, such as described by Di Leonardo *et al*. (44), caused by treatment with DB[*a*,*l*]P or (+)-*syn*- and (–)-*anti*-DB[*a*,*l*]PDE (Figures 5 and 6).

The present study demonstrates that there is a dose-dependent increase in p53 protein levels in MCF-7 cells after exposure to (+)-*syn*- or (–)-*anti*-DB[*a*,*l*]PDE. Both stereoisomeric compounds are the DNA-binding products of metabolic activation of DB[*a*,*l*]P. The stereoisomer with *R*,*S*,*S*,*R*-configuration, the (–)-*anti*-DB[*a*,*l*]PDE, forms significantly more DNA adducts and induces an increase of p53 at significantly lower concentrations than the (+)-*syn*-DB[*a*,*l*]PDE with *S*,*R*,*S*,*R*-configuration. These results together with our previous findings on (+)-*anti*-B[*a*]PDE-treated MCF-7 cells (19) indicate that the increase of wild-type p53 protein levels is related to formation of a critical level of adducts rather than by specific adduct structures and configurations. The results demonstrate that formation of DB[*a*,*l*]PDE–DNA adducts also causes a long-term induction of the CDK inhibitor $p21^{WAF1}$. The presence of an increase in $p21^{WAF1}$ protein levels in the absence of a detectable increase in p53 protein levels in cells treated with 0.01 μM (+)-*syn*-DB[*a*,*l*]PDE suggests that p53-independent induction of $p21^{WAF1}$ can also result from DB[*a*,*l*]PDE–DNA adduct formation. The long term induction of $p21^{WAF1}$ after formation of DB[*a*,*l*]PDE–DNA adducts may lead to G_2/M arrest, which remains to be further established.

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