The Role of the Ah Receptor and p38 in Benzo[a]pyrene-7,8-dihydridiol and Benzo[a]pyrene-7,8-dihydridiol-9,10-epoxide-induced Apoptosis*

Shujuan Chen, Nghia Nguyen, Kumiko Tamura‡, Michael Karin‡, and Robert H. Tukey§

From the Laboratory of Environmental Toxicology, Departments of Pharmacology, Chemistry & Biochemistry and the ‡Laboratory of Gene Regulation, Department of Pharmacology, University of California, San Diego, La Jolla, California 92093-0636

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in the environment. Benzo[a]pyrene (B[a]P), a prototypical member of this class of chemicals, affects cellular signal transduction pathways and induces apoptosis. In this study, the proximate carcinogen of B[a]P metabolism, trans-7,8-dihydroxy-7,8-dihydridiol [B[a]P 7,8-dihydridiol] and the ultimate carcinogen, B[a]P-r-7,8-dihydridiol-9,10-epoxide(±) (BPDE-2) were found to induce apoptosis in human HepG2 cells. Apoptosis initiated by B[a]P-7,8-dihydridiol was linked to activation of the Ah receptor and induction of CYP1A1, an event that can lead to the formation of BPDE-2. With both B[a]P-7,8-dihydridiol and BPDE-2 treatment, changes in anti- and pro-apoptotic events in the Bcl-2 family of proteins correlated with the release of mitochondrial cytochrome c and caspase activation. The onset of apoptosis as monitored by caspase activation was linked to mitogen-activated protein (MAP) kinases. Utilizing mouse hepalc1c7 cells and the Arnt-deficient BPRc1 cells, activation of MAP kinase p38 by B[a]P-7,8-dihydridiol was shown to be Ah receptor-dependent, indicating that metabolic activation by CYP1A1 was required. This was in contrast to p38 activation by BPDE-2, an event that was independent of Ah receptor function. Confirmation that MAP kinases play a critical role in BPDE-2-induced apoptosis was shown by inhibiting caspase activation of poly(ADP-ribose)polymerase 1 (PARP-1) by chemical inhibitors of p38 and ERK1/2. Furthermore, mouse embryo p38± fibroblasts were shown to be resistant to the actions of BPDE-2-induced apoptosis as determined by annexin V analysis, cytochrome c release, and cleavage of PARP-1. These results confirm that the Ah receptor plays a critical role in B[a]P-7,8-dihydridiol-induced apoptosis while p38 MAP kinase links the actions of an electrophilic metabolite like BPDE-2 to the regulation of programmed cell death.

Benzo[a]pyrene (B[a]P) is a representative polycyclic aromatic hydrocarbon (PAH) that is generated as a result of combustion and is found in significant concentrations in tobacco smoke (1). As a ubiquitous environmental contaminant B[a]P is formed as a byproduct of industrialization with traces identified in air-borne particles (2), water supplies, as well as food and dietary sources (3). Experiments in animals convincingly demonstrate that B[a]P exposure leads to the generation of tumors (4–6) and has been implicated as a human carcinogen. Benzo[a]pyrene is a procarcinogen requiring metabolism and metabolic activation by cytochrome P450 (CYP)-dependent oxidations and epoxide hydrolysis to form the ultimate carcinogens (7). Tumorigenicity studies have demonstrated that the product generally accepted as the ultimate carcinogen of B[a]P metabolism is the anti or trans isomer of B[a]P-r-7,8-dihydridiol-9,10-epoxide (BPDE-2) (8, 9), which can bind to DNA (10–12) and serve as an initiator of carcinogenesis (1, 13). The formation of BPDE-2 results from cytochrome P450-dependent oxidation of B[a]P to B[a]P-7,8 oxide, followed by hydration by epoxide hydrolase to B[a]P-7,8-dihydridiol, which then serves as a substrate for a second CYP-dependent oxidation reaction generating BPDE-2. In tissue culture experiments, B[a]P-7,8-dihydridiol and BPDE-2 have been shown to initiate programmed cell death or apoptosis (14). Thus, the onset of apoptosis by procarcinogens like B[a]P may be viewed in its simplest form to proceed in two independent steps. The first step requires cell-specific metabolism by oxidative and hydrolytic enzymes generating the ultimate carcinogen, and the second step requires the involvement of cellular events that define cell death.

Whereas several P450s are capable of metabolizing B[a]P, the oxidation reaction that most efficiently generates B[a]P-dependent mutagenesis is catalyzed by CYP1A1 (15–17) and CYP1B1 (18, 19). Expressed CYP1A1 and CYP1B1 display similar catalytic activities in converting B[a]P-7,8-dihydridiol to mutagenic metabolites (20). Induction of Cyp1a1 and Cyp1b1 in wild-type and Ah receptor-deficient mice by polycyclic aromatic hydrocarbons and polychlorinated biphenyls confirmed that expression of Cyp1a1 and Cyp1b1 is dependent upon the Ah receptor (21). Induced by PAHs following activation of the Ah receptor, CYP1 proteins catalyze the formation of B[a]P-oxide as well as the activation of B[a]P-7,8-dihydridiol to the mutagenic BPDE-2 metabolite. Benzo[a]pyrene is an inducer of the CYP1A1 gene (22) and has been shown to stimulate apoptosis in mouse hepalc1c7 hepatoma cells (23) and Daudi human B cells (24). Thus, since apoptosis by B[a]P is dependent

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§ To whom correspondence should be addressed: Dept. of Pharmacology, Chemistry & Biochemistry, UCSD, La Jolla, CA 92093-0636. Tel.: 858-582-0288; Fax: 858-582-0363; E-mail: rtukey@ucsd.edu.

‡ Laboratory of Gene Regulation, Department of Pharmacology, University of California, San Diego, La Jolla, California 92093-0636.
been speculated that consumption of nicotinamide adenine dinucleotide (NAD) involved in DNA repair and transcription (28). PARP-1 activation initiated by DNA damage leads to the promotion of DNA repair and the activation of poly(ADP-ribose) polymerase-1 (PARP-1) (27), which transfers ADP-ribose to other nuclear proteins involved in DNA repair and transcription (28). PARP-1 activation is also felt to be a mediator of cell death. While the actual mechanism of PARP-1 induced cell death is unknown, it has been speculated that consumption of nicotinamide adenine dinucleotide (NAD\(^+\)) used in PARP-1-initiated ADP-ribosylation leads to depletion of NAD\(^+\) and the eventual disruption of mitochondrial function, an event that stimulates cytochrome c release and caspase activation (29). Results have indicated that PARP-1, a substrate for caspases, is targeted for cleavage and inactivated during apoptosis, possibly disrupting poly(ADP-ribose)ylation and eventually allowing for the promotion of nuclear disintegration by endonucleases. In Daudi Human B cells, treatment with B[a]P and B[a]P-7,8-dihydrodiol resulted in DNA fragmentation and the cleavage of PARP-1 (24), indicating that DNA damage may be the leading initiator of apoptosis.

Central to the events leading to apoptosis are the role of cellular signaling pathways in controlling programmed cell death, in particular the phosphorylation cascades that regulate MAP kinases (30). The MAP kinases include extracellular signal-related kinase (ERK), c-Jun NH\(_2\)-terminal protein kinase (JNK), and p38 kinase. Carbon black particles containing B[a]P have been shown to stimulate the release of tumor necrosis factor \(\alpha\) resulting in regulation of MAP kinase activity, a proposed mechanism to induce apoptosis in RAW 264.7 macrophage cells (31). Exposure of 293T and HELa cells with B[a]P was shown to up-regulate \(\alpha\)-PAK-exchange factor in a manner concordant with activation of JNK1, a finding that was tied to B[a]P induction of caspase-mediated apoptosis (32). It has also been concluded that oxidative stress induced cell death is regulated in part by p38 and caspase 8 activation, with singlet oxygen-activating p38 upstream of caspase 8-dependent cleavage of Bid (33). A role for p38 activation in the apoptotic pathway has also been implicated with agents that lead to DNA damage (34), a finding that may suggest an important role for p38 in B[a]P-induced cell death since BPDE-2 is genotoxic.

In this study, we examined the relationship between the Ah receptor and signal transduction pathways in B[a]P-7,8-dihydrodiol- and BPDE-2-induced apoptosis. An approach is outlined that uses human HepG2 cells, mouse hep1c1c7 and Arnt-defective BPCr1 cells (35) to examine the role of the Ah receptor in both MAP kinase activation and cell death initiated by B[a]P-7,8-dihydrodiol and BPDE-2. Apoptosis is shown to be linked to p38 activation using mouse embryo fibroblasts (MEF) obtained from p38-deficient mice, indicating that p38 plays an important role in BPDE-2 initiated cell death.

**MATERIALS AND METHODS**

**Chemicals and Reagents—**trans-7,8-dihydroxy-7,8-dihydrobenzo-[a]pyrene (B[a]P-7,8-dihydrodiol), Benzo[a]pyrene-7,8-dihydrorylon-t-9,10-epoxide (\(\times\)) (BPDE-2) were purchased from NCI, Chemical Carcinogens Repositories (National Institutes of Health, Bethesda, MD). All chemicals were dissolved in Me\(_2\)SO or tetrahydrofuran. The final concentration of Me\(_2\)SO or tetrahydrofuran in cell cultures was 0.1%. Mitogen-activated protein kinase inhibitors, PD 98059, SB 203580, and U0126 were purchased from Calbiochem (San Diego, CA) and dissolved in Me\(_2\)SO to appropriate concentrations. All other chemicals were obtained through standard suppliers. The mono- or polycyclic primary antibodies, anti-human/mouse phosphorylated p38, phosphorylated ERK1/2, p38, ERK, and anti-human Bid were purchased from Cell Signaling (Beverly, MA). Anti-human Bcl-x\(_L\) was from Signal Transduction (San Jose, CA), anti-human/mouse cytochrome c and PARP were from BD Pharmingen (San Diego, CA), anti-human/mouse Bak was from Upstate (Waltham, MA). Rabbit anti-human CYP1A1 (36) was a generous gift from Dr. Fred Guengerich, Vanderbilt University.

**Cell Culture—**The human hepatoma cell line, HepG2, was obtained from the American Type Culture Collection. TV101L cells were developed in this laboratory from HepG2 cells and stably express a CYP1A1 luciferase reporter gene (22). TV101L cells were grown under the same conditions as HepG2 cells but with the addition of 0.8 mg/ml A8. Wild-type mouse hep1c1c7 and Arnt-defective BPCr1 cells (35) were a generous gift from Dr. James Whitlock, Stanford University. Wild-type MEF and those deficient in p38 have been described previously (37). Transient transfection of BPCr1 cells with a full-length human Arnt cDNA (pArnt/CMV4) was conducted as previously described (38). All cell lines in this study were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and supplemented with penicillin/streptomycin (10,000 units/ml). Cells were incubated in a humidified incubator under 5% CO\(_2\) at 37 °C.

**Apoptosis Assay—**Detection of apoptotic oligonucleosomal DNA fragmentation was performed as described (39). Approximately 1 × 10\(^6\) HepG2 cells were exposed to different concentrations of B[a]P-7,8-dihydrodiol for 24 h and the cells collected by trypsinization and pelleted at 1,000 × g for 5 min. Cell pellets were resuspended in 55 μl of lysis buffer (20 μM EDTA, 10 μM Tris-HCl, pH 8.0, 0.8% SDS) and treated with 20 μl RNase A (10 μg/ml) at 37 °C for 1 h, followed by the addition of 25 μl of proteinase K (20 mg/ml) and then incubated at 55 °C overnight. Lysates were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and DNA precipitated with 2 volumes of ice-cold absolute ethanol. The DNA was collected in a microcentrifuge and resuspended in 20 μl of 10 mM Tris-HCl, pH 8.0, 10 μM EDTA. DNA samples were subjected to electrophoresis in 1.5% agarose gels.

**Confirmation of apoptosis was quantified by measurement of externalized phosphatidylserine residues as detected using annexin V-FITC (BD Pharmingen). After exposure to appropriate concentrations of chemicals, cells were collected and washed with ice-cold phosphate-buffered saline and then suspended in 500 μl of annexin V binding buffer. A 500-μl aliquot was taken, 5 μl of annexin V-FITC was added, and the mixture incubated for 15 min at room temperature in the dark. After the addition of 400 μl of binding buffer, the cells were acquired on a FACS Calibur flow cytometer and analyzed using CELLQuest software. The results are shown as a histogram with annexin V-positive cells calculated as apoptotic cells.**

**Cell Viability Assay (MTT Assay)—**Cell viability was assessed by 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay (40). After treatment with various concentrations of appropriate chemicals, the culture medium was replaced with serum-free medium containing 0.5 mg/ml MTT, and cultures were incubated for...
an additional 3 h. The blue MTT formazan was dissolved in 1 ml of isopropanol alcohol with 0.04% HCl, and the absorbance values were determined at a 570-nm test wavelength and a 630-nm reference wavelength using a DU 640B spectrophotometer (Beckman Coulter). The results are displayed as percent of viable cells compared with the vehicle control.

**Western Blot Analysis**—All Western blots were performed using Nu-PAGE Bis-Tris gel electrophoresis as outlined by the supplier (Invitrogen). For total cellular protein, cells were lysed in buffer containing 25 mM Hepes, pH 7.5, 0.3 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.5 mM DTT, 1 mM sodium orthovanadate, 0.1 mM okadaic acid, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations of the cell lysates were determined by Bio-Rad analysis according to the manufacturer’s instruction. A 30-μg aliquot was boiled for 5 min in loading buffer and resolved on a 10% Bis-Tris gel under denaturing conditions and the proteins transferred to polyvinylidene difluoride membrane using a semidry transfer system (Norvex). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline for 1 h at room temperature, followed by incubation with primary antibodies in Tris-buffered saline overnight at 4 °C. Membranes were washed and exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Each membrane was again washed, and the conjugated horseradish peroxidase was detected using the ECL Plus Western blotting detection system (Amersham Biosciences) and scanned with a Molecular Dynamics Storm 840 scanner.

For Western blot analysis of microsomal CYP1A1, cells were scraped from the plates and suspended in 0.25% sucrose (1.5, v/v). Cell suspensions were homogenized 20 times in a Kontes Potter-Elvehejm tissue grinder and the suspensions centrifuged at 5,000 × g in a Sorvall RT 6000B-refrigerated centrifuge. The supernatant was collected and centrifuged at 150,000 × g for 1 h in a Beckman TL-100 tabletop ultracentrifuge. The microsomal pellet was suspended in 500 μl of 50 mM Tris-Cl, pH 7.4, 10 mM MgCl₂, and 1 mM phenylmethylsulfonyl fluoride. A 10-μg aliquot was processed by Western blot analysis as outlined above. Detection of CYP1A1 was performed using a rabbit anti-human CYP1A1 antibody.

**Cytochrome c Release Analysis**—The conditions used for cytochrome c release have been outlined (41). Cells were collected and incubated in 1 ml of isotonic buffer containing 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride with protease inhibitors. After 15 min on ice, the cells were homogenized and then centrifuged at 1,000 × g for 10 min at 4 °C in a microcentrifuge. The supernatant was centrifuged at 15,000 × g for 15 min in a microcentrifuge, followed by a final centrifugation at 100,000 × g for 1 h in a Beckman TL-100 tabletop centrifuge. The resulting supernatant (S-100) was stored at −80 °C and used for Western blot analysis.

**RT-PCR for CYP1A1 Gene Transcripts**—Total RNA was extracted from cells using acidic phenol/quainidium isothiocyanate solution (TRizol, Invitrogen). 3 μg of total RNA was denatured together with oligo(dT) primer at 70 °C for 10 min. The synthesis of cDNA has been outlined (42).

For amplification of CYP1A1, two primers were generated. The forward and reverse primers were obtained from DNA sequence (accession number AF253322) of the CYP1 locus (43). The forward primer was 5′-GGTGGTGTGCTGAGCCGG-3′ (bases 7661–7680) and the reverse primer was 5′-CCTGCCAGGGGCTTGGC-3′ (bases 5822–5842). In a reaction volume of 96 μl containing 3 μl MgCl₂, 50 μl KCl, 20 μl Tris-Cl, pH 8.4, and 0.2 μmol of each dNTP, 2 μmol of each primer, and 5 units of VENT (exo-) DNA polymerase, cycling was carried out at 94 °C for 1 min, 59 °C (60 s), 59 °C (60 s), and 72 °C (60 s) for 35 cycles. The protocol was preceded by an incubation of 5 min at 95 °C and followed by an extended elongation time of 7 min at 72 °C.

**Luciferase Activity Assay**—Luciferase assays were carried out as previously described (44). TV101L cells were treated and lysed on plates in a buffer containing 1% Triton, 25 mM Tricine, pH 7.8, 15 mM MgSO₄, 4 mM EDTA, and 1 mM DTT. Cell lysates were centrifuged at 14,000 × g in a microcentrifuge for 10 min at 4 °C, and supernatants were used for luciferase and protein assays. A cell extract aliquot of 10 μl was mixed with 300 μl of reaction mixture, which contained 15 mM potassium phosphate buffer, pH 7.8, 15 mM MgSO₄, 2 mM ATP, 4 mM EDTA, 25 mM Tricine, and 1 mM DTT. Reactions were started by adding 100 μl of luciferin (0.3 mg/ml) and light output measured for 10 s at 24 °C using a Monolight 2001 luminometer (Analytical Luminescence Laboratory). The results were normalized by protein concentrations and expressed as fold induction of vehicle control.

**RESULTS**

**B[a]P-7,8-dihydriodiol and BPDE-2 Initiate Apoptosis in HepG2 Cells**—DNA fragmentation and mitochondrial release of cytochrome c (41) are well characterized biochemical markers of apoptosis. The treatment of HepG2 cells with B[a]P-7,8-dihydriodiol induced apoptosis in a dose-dependent fashion as demonstrated by DNA fragmentation (Fig. 1A), as well as cytochrome c release (Fig. 1B). Other key regulatory proteins involved in controlling cytochrome c release are the Bcl-2 family of proteins, some of which promote cell survival such as Bcl-2, or induce cell death, such as Bax and Bid. Early markers of apoptosis are characterized by the activation of caspase 8 by death receptors and the resulting cleavage and activation of the pro-apoptotic protein Bid (45). B[a]P-7,8-dihydriodiol treatment leads to Bid cleavage as demonstrated in Fig. 1C. Concordant with Bid cleavage are increases in the pro-apoptotic Bak protein (Fig. 1C), which has been shown to accelerate cytochrome c release (45). In addition, B[a]P-7,8-dihydriodiol stimulates a reduction in anti-apoptotic proteins such as Bcl-xL (Fig. 1C), which serves to resist mitochondrial release of cytochrome c. Thus, changes in the levels of expression of the Bcl-2 family of proteins by B[a]P-7,8-dihydriodiol is consistent with the observed accumulation of cytosolic cytochrome c.

Apoptosis is executed through the activation of caspasess by cytochrome c (46). To examine if the increases in DNA frag-
mitochondrial respiration resulting in mitochondrial disrup-
tion and mitochondrial cytochrome c release. Polyclonal antibodies to PARP-1 (Fig. 1B) were used to detect cleavage of PARP-1, which is a marker of apoptosis. The treatment of HepG2 cells with increasing concentrations of BPDE-2 leads to cleavage of PARP-1, concordant in a dose-dependent manner. The treatment of HepG2 cells with BPDE-2 at concentrations comparable to those observed in vivo resulted in cleavage of PARP-1, indicating apoptosis.

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viability and PARP-1 cleavage in hepa1c1c7 and BPRc1 cells. A
served at 2.5 and 10 μM BaP-7,8-dihydrodiol (BP-7,8-diol) or BPDE-2. The differences ob-

bined on mouse hepa1c1c7 and BPRc1 cells after treatment for 24 h with different concentrations of BaP-7,8-dihydrodiol (BP-7,8-diol) or BPDE-2. The differences observed at 2.5 and 10 μM BaP-7,8-dihydrodiol between hepa1c1c7 and BPRc1 cells (figure on the left) is statistically significant (p < 0.01, Student’s t test). B, Western blot analysis of PARP-1 cleavage in hepa1c1c7 and BPRc1 cells, and BPRc1 cells transfected with pArnt/CMV4 following treatment with different concentrations of BaP-7,8-dihydrodiol. C, Western blot analysis of PARP-1 cleavage in hepa1c1c7 and BPRc1 cells treated with different concentrations of BPDE-2. Total cell extracts were used for analysis of activated PARP-1.

FIG. 4. The effect of B[a]P-7,8-dihydrodiol and BPDE-2 on cell viability and PARP-1 cleavage in hepa1c1c7 and BPRc1 cells. A, cell viability assays (MTT) were conducted on mouse hepa1c1c7 and BPRc1 cells. B, Western blot analysis of PARP-1 cleavage in hepa1c1c7 and BPRc1 cells, and BPRc1 cells transfected with pArnt/CMV4 following treatment with different concentrations of B[a]P-7,8-dihydrodiol. C, Western blot analysis of PARP-1 cleavage in hepa1c1c7 and BPRc1 cells treated with different concentrations of BPDE-2. Total cell extracts were used for analysis of activated PARP-1.

national Cyp1a1 in microsomes from B[a]P-7,8-dihydrodiol-treated cells, was only detected in hepa1c1c7 cells (data not shown). These results demonstrate that BPRc1 cells are unable to accumulate functional Cyp1a1 through activation of the Ah receptor by B[a]P-7,8-dihydrodiol.

Confirmation that Ah receptor activation and accumulation of Cyp1a1 in response to B[a]P-7,8-dihydrodiol treatment is linked to apoptosis was examined by analysis of cell viability and the initiation of apoptosis in hepa1c1c7 and BPRc1 cells. When cell viability was determined by the MTT assay, B[a]P-7,8-dihydrodiol treatment of hepa1c1c7 and BPRc1 cells demonstrated that BPRc1 cells were refractory to cell death (Fig. 4A). These results indicate that BPRc1 cells were also resistant to B[a]P-7,8-dihydrodiol-induced apoptosis, which was demonstrated by a lack of PARP-1 cleavage in BPRc1-treated cells (Fig. 4B). When BPRc1 cells were transfected with a human Arnt cDNA expression plasmid (47) to reverse the genetic defect in Arnt expression, the treatment of these cells with B[a]P-7,8-dihydrodiol-induced apoptosis as measured by PARP-1 cleavage. The initiation of apoptosis by B[a]P-7,8-dihydrodiol requires a functional Ah receptor/Arnt complex, a requirement that promotes CYP1A protein induction and accelerates the production of DNA damaging metabolites like BPDE-2. While metabolism of B[a]P-7,8-dihydrodiol is clearly a requirement, an electrophilic metabolite like BPDE-2 induces the apoptotic event independent of the Ah receptor. This is demonstrated in Fig. 4C where BPDE-2 initiates apoptosis in both wild-type hepa1c1c7 and Arnt-deficient BPRc1 cells.

Evidence for MAP Kinases and Apoptosis—Several central cellular pathways known to be involved in controlling apoptosis and cell death are MAP kinase modules (48). HepG2 cells treated with B[a]P-7,8-dihydrodiol activated both p38 and ERK1/2 as demonstrated by an increase in the phosphorylated form of these proteins (Fig. 5A). Similar patterns of MAP kinase activation occur when HepG2 cells are treated with BPDE-2 (data not shown). In addition, the activation of p38 by B[a]P-7,8-dihydrodiol is dependent upon a functional Ah receptor, as demonstrated by the phosphorylation of p38 with B[a]P-7,8-dihydrodiol in hepa1c1c7 cells but not BPRc1 cells. Since BPDE-2 induces the phosphorylation of p38 in both hepa1c1c7 and BPRc1 cells (Fig. 5B), it can be concluded that the ultimate carcinogen is responsible for MAP kinase activation, while the Ah receptor is essential in promoting MAP kinase activation following exposure to the proximate carcinogen B[a]P-7,8-dihydrodiol.

Since BPDE-2 leads to apoptosis in HepG2 cells, we examined the actions of cellular inhibitors of p38 and ERK1/2 for their ability to block cell death as measured through caspase-dependent cleavage of PARP-1 (Fig. 5C). SB203580 is a p38 inhibitor while PD98059 and U0126 are ERK1/2 inhibitors. Inhibition of MAP kinase activity by SB203580, PD98059, and U0126 blocked BPDE-2-induced apoptosis as determined by
Phospho-p38

p38+/+
p38−/−

p38+/+
p38−/−

p38+/+
p38−/−

Fig. 6. Analysis of BPDE-2-induced apoptosis in p38-deficient MEFs. Wild-type MEFs (p38+/+) and p38-deficient MEFs (p38−/−) were treated with different concentrations of BPDE-2 for 24 h. Analysis of p38 activation in p38+/+ and p38−/− cells by Western blot analysis. B, shown in this figure are the results of annexin V-FITC analysis. Represented in each box are the percentiles of cells that are apoptotic. C, analysis of PARP-1 cleavage and cytochrome c release as a function of p38 phenotype as monitored by Western blot analysis.

PARP-1 cleavage (Fig. 5C). The observation that MAP kinase inhibitors interfere with BPDE-2-induced caspase-dependent apoptosis strongly suggests that MAP kinases such as p38 play an important role in BPDE-2-initiated apoptosis.

Role of p38 in BPDE-2-initiated Apoptosis—To examine the contribution of p38 in BPDE-2-induced apoptosis, p38+/+ and p38−/− mouse embryo fibroblasts (MEFs) were treated with BPDE-2 and apoptosis monitored. Treatment of p38+/+ MEFs with BPDE-2 resulted in p38 activation while this activity was not detectable in p38−/− MEFs (Fig. 6A). BPDE-2-induced apoptosis was linked to p38 as shown by annexin V FITC analysis (Fig. 6B). Cells treated with different concentrations of BPDE-2 and analyzed by flow cytometry demonstrated that BPDE-2-treated p38−/− MEFs displayed greatly reduced apoptotic cells when compared with BPDE-2-treated p38+/+ MEFs. The reduction in BPDE-2-induced apoptosis in p38−/− MEFs corresponded to a lack of cytochrome c release and a similar resistance to caspase-dependent cleavage of PARP-1 (Fig. 6C).

DISCUSSION

B[a]P-7,8-dihydrodiol undergoes CYP-dependent epoxidation to form the ultimate carcinogen, BPDE-2. In these studies, we demonstrate that cellular events leading to caspase activation that include activation of MAP kinases serve as the core apparatus leading to programmed cell death (49). Intracellular DNA fragmentation, evident from exposure of HepG2 cells to B[a]P-7,8-dihydrodiol, is the terminal step in disposal of the genome in cells undergoing apoptosis and is known to follow caspase-dependent activation of DNase (50). Examination of the signaling steps encoding programmed cell death in HepG2 cells resulting from B[a]P-7,8-dihydrodiol and BPDE-2 exposure demonstrates that apoptosis proceeds through cytochrome c release from mitochondria, a prerequisite for activation of caspases (41). We have demonstrated that several key regulatory steps involved in cytochrome c release are activated in response to B[a]P-7,8-dihydrodiol exposure. For example, the integrity of the mitochondria is in part controlled by pro-apoptotic and anti-apoptotic proteins of the Bel-2 family. In HepG2 cells, exposure to B[a]P-7,8-dihydrodiol initiates degradation of Bid and the activation of other pro-apoptotic proteins such as Bak. Simultaneously, B[a]P-7,8-dihydrodiol stimulates the inhibition of anti-apoptotic proteins such as Bel-xL, which serves to inhibit mitochondrial cytochrome c release. Along with results demonstrating that caspase-specific substrates such as PARP-1 are cleaved following B[a]P-7,8-dihydrodiol and BPDE-2 treatment of hepatoma cells, it is clear that mitochondrial cytochrome c release and caspase activation play a prominent role in the actions of these carcinogens in programmed cell death.

B[a]P-7,8-dihydrodiol is a primary metabolite of B[a]P and is considered a proximate carcinogen requiring metabolic activation to the ultimate carcinogen BPDE-2. Because B[a]P-7,8-dihydrodiol is a weak mutagen, apoptosis following B[a]P-7,8-dihydrodiol treatment requires metabolic activation. With no detectable CYP1A1 protein in HepG2 cells, we demonstrated that B[a]P-7,8-dihydrodiol is capable of inducing CYP1A1. B[a]P-7,8-dihydrodiol treatment of HepG2 cells leads to transcriptional activation of the CYP1A1 gene as confirmed by activation of the CYP1A1-luciferase gene in TV101 cells and induction of both CYP1A1 RNA and protein. Conclusive evidence that B[a]P-7,8-dihydrodiol induces CYP1A1 in an Ah receptor-dependent manner was demonstrated using wild-type and Ah receptor complex defective mouse hepatoma cells. The observation that B[a]P-7,8-dihydrodiol induces Cyp1a1 in Hepa1c1c7 cells but not in the Arnt defective BPRc1 cells demonstrates that B[a]P-7,8-dihydrodiol is an Ah receptor agonist. In addition, it can be concluded that the events leading to B[a]P-7,8-dihydrodiol-induced apoptosis as monitored through DNA fragmentation and cytochrome c release in HepG2 cells also requires a functional Ah receptor complex (AhR/Arnt), since cell death as monitored by cell viability and apoptosis as shown with PARP-1 cleavage is not apparent in the Ah receptor-deficient BPRc1 cells. Thus, B[a]P-7,8-dihydrodiol can stimulate its own metabolism through activation of the Ah receptor and induction of CYP1A1, leading to the ultimate carcinogen BPDE-2. Since B[a]P also activates the Ah receptor, it is difficult to speculate on the total contribution of B[a]P-7,8-dihydrodiol toward Ah receptor activation and CYP1 gene expression. However, the observation that major B[a]P metabolites serve as ligands for the Ah receptor can be considered a cellular mechanism for facilitating elevated levels of activated Ah receptor, a result that provides the cell with an abundance of CYP1 gene products.

With metabolism of B[a]P-7,8-dihydrodiol to the mutagenic BPDE-2 being mediated through Ah receptor activation, one can only speculate on a cellular mechanism that initiates caspase activation and apoptosis. Since BPDE-2 is a known mutagen and DNA-damaging agent, the involvement of PARP-1 in the suicide hypothesis (51) may underlie B[a]P-induced cell death. PARP-1 metabolizes β-nicotinamide adenine dinucleotide (NAD+) into polymers of ADP-ribose that are then transferred to nuclear proteins involved in DNA repair.
and chromatin binding as well as other genomic processes such as DNA base excision repair, DNA replication, and transcription (28). While DNA repair and PARP-1 activation is a normal process, events leading to the overactivation of PARP-1 and the depletion of NAD⁺ from the mitochondria can impair NAD⁺-dependent metabolic processes such as glycolysis and mitochondrial respiration, eventually reducing ATP production and cellular function. Such a mechanism could be envisioned to result in the collapse of the mitochondria and the release of cytochrome c, an event that is necessary for the initial events leading to caspase activation. PARP-1 is a substrate for multiple caspases (52), and the degradation of PARP-1 results in the loss of nick-sensor function and is thus inactive toward DNA damage. PARP-1 has been postulated to be involved in the regulation of the endonuclease activity of the DNAs1L3 (53, 54), which is inhibited during poly(ADP-ribosyl)ation activation. Thus, loss of PARP-1 activity through caspase activation has been speculated to release endonuclease inhibition allowing for the unprogrammed cleavage of DNA.

Bid plays an important role in promoting the apoptotic machinery following its activation by caspase 8 and translocation to the mitochondria (45, 46), where it stimulates a conformational change in the pro-apoptotic Bax protein leading to cytochrome c release (45). Our results also suggest that Bid, a BH3 domain containing protein, is activated in HepG2 cells following treatment with B[a]P-7,8-dihydrodiol. The initiating step leading to the cleavage of Bid by caspase 8 is triggered by interaction of tumor necrosis family (TNF) death ligands with their corresponding death receptors. Death receptors such as CD95, Fas, and Apo1 are part of the TNF receptor family, characterized by a cysteine-rich extracellular domain and a cytoplasmic domain, which has been termed the death domain (reviewed in Ref. 55). The death domains are responsible for triggering the apoptotic machinery. Death receptors associate with adaptor proteins such as FADD (f-as-associated death domain), which in turn leads to binding and activation of caspase-8. Caspase-8 can activate downstream caspases such as caspase-9, in addition to the cleavage and activation of Bid.

Thus, we expect that the treatment of HepG2 cells with B[a]P-7,8-dihydrodiol leads to the activation of caspase-8, since Bid is subject to proteolytic cleavage. However, our results do not directly link the actions of death receptors in B[a]P-7,8-dihydrodiol-induced apoptosis. Recent experiments examining the action of the PAH compound 7,12-dimethylbenz[a]anthracene on pre-B cell apoptosis indicates that caspase-8 activation may be independent of death receptor ligation (56).

While B[a]P-7,8-dihydrodiol and BPDE-2 are capable of initiating programmed cell death, B[a]P-7,8-dihydrodiol requires both the Ah receptor and p38 while the ultimate carcinogen BPDE-2 is dependent only upon p38 activation. MAP kinase p38 has been implicated in apoptosis through multiple insults including nitric oxide (57), retinoid-related molecules (58), 3-methylcholanthrene (59), and tamoxifen (60), to name a few. We have demonstrated that the Ah receptor and p38 are central to the processes of B[a]P-7,8-dihydrodiol-induced apoptosis. The Ah receptor serves as a target for PAHs like B[a]P and its metabolites and its activation is a prerequisite for further metabolism to agents that initiate programmed cell death. This was demonstrated when B[a]P-7,8-dihydrodiol was unable to activate p38 or stimulate apoptosis in Arnt-deficient cells, but efficiently activate the Ah receptor in wild-type cells leading to induction of Cyp1a1 and activation of p38. However, BPDE-2, the ultimate metabolite of Cyp1-directed B[a]P-7,8-dihydrodiol metabolism, activates p38 and stimulates apoptosis in an Ah receptor-independent fashion.

While metabolites of B[a]P-7,8-dihydrodiol like BPDE-2 initiate programmed cell death with a strict requirement for p38 activation, the cellular processes that result in p38 activation, and the role of p38 in controlling apoptosis is unknown. MAP kinase activation results from external stimuli with the resulting signaling effects leading to regulation of gene transcription as well as phosphorylation of substrates in the cytoplasm and other subcellular compartments (61). For example, p38 MAP kinase activity plays an important role in nitric oxide-mediated cortical neuron cell death by stimulating Bax translocation to the mitochondria and activating cell death pathways (62). However, regulation of gene transcription by p38 is best associated with apoptosis (61). From our results, we can speculate that the role of activated p38 in BPDE-2-induced apoptosis must be influencing cellular control prior to mitochondrial cytochrome c release and caspase activation, as demonstrated by the inability of BPDE-2 to stimulate apoptosis, cytochrome c release and PARP-1 cleavage in p38−/− MEFs. It has been shown that genotoxic stress stemming from chemotherapeutic agents leads to p38 activation, which directly phosphorylates and activates p53 (34), a transcriptional factor that has been implicated in promoting apoptosis. Since MAP kinase activity in the nucleus results in the activation of transcriptional factors, most of which are already bound to DNA, BPDE-2 exposure to cells may be triggering through a p38-dependent mechanism those genes that are necessary for programmed cell death.

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The Role of the Ah Receptor and p38 in Benzo[a]pyrene-7,8-dihydriodiol and Benzo[a]pyrene-7,8-dihydriodiol-9,10-epoxide-induced Apoptosis

Shujuan Chen, Nghia Nguyen, Kumiko Tamura, Michael Karin and Robert H. Tukey

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