Effects of Polycyclic Aromatic Hydrocarbons (PAHs) on Vascular Endothelial Growth Factor Induction through Phosphatidylinositol 3-Kinase/AP-1-dependent, HIF-1α-independent Pathway*

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Previous studies have demonstrated that exposure to polycyclic aromatic hydrocarbons (PAHs) and its derivatives is associated with an increased risk of skin cancers, and the carcinogenic effect of PAHs is thought to involve both tumor initiation and promotion. Whereas PAH tumor initiation is well characterized, the mechanisms involved in the tumor promotion of PAHs remain elusive. In the present study, we investigated the effects of PAHs on vascular endothelial growth factor (VEGF) expression by comparison of its induction between the active metabolite and its parent compound (B[a]PDE versus B[a]P) or between active compound and its relatively inactive analog (5-MCDE versus CDE). We found that exposure of cells to (+)-anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (B[a]PDE) or (+)-anti-5-methylchrysene-1,2-diol-3,4-epoxide (CDE) did not exhibit significant inductive effects. Exposure of cells to B[a]PDE and 5-MCDE did not induce HIF-1α activation, whereas AP-1 was significantly activated. Moreover, overexpression of TAM67 (a dominant-negative mutant c-Jun) dramatically blocked that VEGF induction. Electrophoretic mobility shift assay (EMSA) expression of TAM67 (a dominant-negative mutant c-Jun) dramatically activated, whereas AP-1 was significantly activated. Moreover, overexpression of TAM67 (a dominant-negative mutant c-Jun) dramatically blocked that VEGF induction. Electrophoretic mobility shift assay (EMSA) expression of TAM67 (a dominant-negative mutant c-Jun) dramatically activated, whereas AP-1 was significantly activated. Moreover, overexpression of TAM67 (a dominant-negative mutant c-Jun) dramatically blocked that VEGF induction. Electrophoretic mobility shift assay (EMSA) expression of TAM67 (a dominant-negative mutant c-Jun) dramatically activated, whereas AP-1 was significantly activated. Moreover, overexpression of TAM67 (a dominant-negative mutant c-Jun) dramatically blocked that VEGF induction.

5-MCDE is through PI-3K/AP-1-dependent and HIF-1α-independent pathways. These findings may help us to understand the mechanisms involved in PAH carcinogenic effects.

Polycyclic aromatic hydrocarbons (PAHs)3 are ubiquitously present in the atmosphere from combustion sources such as cigarette smoking and vehicle emissions as well as industrially through coal tar production.3, 4) Benzo[a]pyrene (B[a]P), for instance, is metabolized into benzo[a]pyrene-7,8-diol-9,10-epoxide (B[a]PDE) (5). B[a]P and its metabolite, B[a]PDE, have been proven to be complete carcinogens in many animal models (6–8). 5-MCDE has also been found to be a strong complete carcinogen (9–11). Therefore, PHAs are important compounds in the etiology of human cancers.

Carcinogenesis is a multistage process that consists of initiation, promotion, and progression (13). Initiation is a rapid and reversible course, whereas promotion is a long term process requiring chronic exposure to a certain compound with tumor promotion activities (14). It is very likely that PAHs contribute to carcinogenesis through involvement at these multiple stages (15, 16). The mutagenic effects of PAHs, which are responsible for tumor initiation, have been extensively documented (5, 9–12). However, their tumor promotion effects, which are mainly mediated by activation of transcription factors, are not well understood. It is thought that the rate-limiting steps in multistage carcinogenesis occur during tumor promotion and tumor progression. Thus, the identification of signaling pathways involved in PAH tumor promotion is not only essential for understanding the tumorigenesis of PAH compounds, but also in providing useful information for tumor chemoprevention. So it is practically important to compare among the parental PAHs, their derivatives as well as their analogs, the potential effects on various signaling pathways and downstream target genes. Because vascular endothelial growth factor (VEGF) is one of the key growth factors involved in carcinogenesis, we here investigate the effects of subgroups of PAHs, including B[a]P and its derivative B[a]PDE, 5-MCDE, and its analog CDE, on VEGF induction, as well as the signaling pathways leading to this induction.

The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; VEGF, vascular endothelial growth factor; AP-1, activator protein-1; HRE, hypoxia-responsive element; B[a]P, benzo[a]pyrene; B[a]PDE, (+)-anti-benzo[a]pyrene-7,8-diol-9,10-epoxide; CDE, chrysene-1,2-diol-3,4-epoxide; 5-MCDE, (+)-anti-5-methylchrysene-1,2-diol-3,4-epoxide; FBS, fetal bovine serum; MEM, Eagle’s minimal essential medium; PI-3K, phosphatidylinositol 3-kinase; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; RT, reverse transcriptase.

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Materials and Methods

Cells and Culture—The mouse epidermal cell line, Cl41, and its transfectants, were cultured in Eagle’s minimal essential medium supplemented with 5% fetal bovine serum (FBS), 2 mM l-glutamine, and 25 μg of gentamicin/ml. Eagle’s MEM was purchased from Calbiochem, and l-glutamine, gentamicin, and FBS were from Invitrogen. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO2. The cultures were dissociated with trypsin and transferred to new 75-cm2 culture flasks (Fisher) two to three times per week. Cl41 AP-1 mass1 are Cl41 cells stably transfected with an AP-1-luciferase reporter described in our previous studies (15–19). Cl41 VEGF mass1 are Cl41 cells stably transfected with a VEGF-luciferase reporter, which was constructed by inserting a 2.65-kb KpnI-BssHII fragment of the human VEGF promoter sequence from −2274 to +379 relative to the transcription initiation site into the pG212-basic vector (Promega) as described previously (20–22). Cl41 HRE mass1 are Cl41 cells stably transfected with a HRE luciferase reporter, which were constructed by inserting the sequence of the HIF-1α binding site into the luciferase reporter vector pG12-b2asic (20, 21). Cl41 VEGF-Luc Δp85 mass1 has been stably cotransfected with the VEGF-luciferase reporter and dominant-negative mutant of p85 as described in our previous report (15).

Reagents and Constructs—The substrate for the luciferase assay was purchased from Promega; B[a]PDE and 5-MCDE were from Eagle-Picher Industries, Inc, Chemsyn Science Laboratories (Lenexa, KS); CDE and 5-MCDE were synthesized. B[a]P, B[a]PDE, CDE, or 5-MCDE was dissolved in Me2SO at 2 mM stock concentration. c-Jun (D) antibody that recognizes the C terminus of c-Jun was purchased from Santa Cruz Biotechnology. The other antibodies used in the studies were purchased from New England BioLabs (Beverly, MA). TAM67 plasmid was a kind gift from Drs. Nancy Colburn and Matthew Young from NCI and Tim Bowden from the University of Arizona.

Generation of Stable Cotransfectants—Cl41 cells were cultured in a 6-well plate until they reached 85–90% confluence. 20 μl of Lipofectamine reagent mixed with 15 μg of plasmids (1 μg of CMV-neo vector, 2 μg of VEGF-luciferase reporter plasmid DNA, 12 μg of TAM67 or vector control) were used to transfect each well in the absence of serum. After 10–12 h, the medium was replaced with 5% FBS MEM. Approximately 30–36 h after the beginning of the transfection, the cells were dissociated with 0.03% trypsin, and cell suspensions were plated into 75-cm2 culture flasks and cultured for 24–28 days with G418 selection (400 μg/ml). The stable clones in a flask were designed as a mass culture (as a stable pool). The stable transfectants were identified by measuring both the basal level of luciferase activity and the inhibition of c-Jun phosphorylation. Stable transfectants, Cl41 VEGF mass1, and Cl41 VEGF TAM67 mass1, were established and cultured in G418-free MEM for at least two passages before each experiment.

VEGF Gene Reporter Assay—Confluent monolayers of Cl41 VEGF-Luc mass1 were trypsinized, and 8 × 104 viable cells suspended in 100 μl of 5% FBS MEM were added to each well of a 96-well plate. The plate was incubated at 37 °C in a humidified atmosphere of 5% CO2 until the cell density reached 80–90%. The cells were then exposed to various PAH compounds for VEGF induction at concentrations and time periods as indicated in the figure legends. The cultures were extracted with 50 μl of lysis buffer, and luciferase activity was measured using the Promega luciferase assay reagent with a luminometer (Wallac 1240 Victor2 multiplateable counter system). The results were expressed as VEGF-luciferase induction relative to control medium containing 0.1% Me2SO (relative VEGF-luciferase induction).

AP-1- and HRE-dependent Transcriptional Activation Assay—The same procedure as described above was used to determine the effects of
PAHs on AP-1- and HRE-dependent transcriptional activation using Cl41 AP-1 mass1 and Cl41 HRE mass1. The results were expressed as AP-1 or HRE activity relative to control medium containing 0.1% Me2SO (relative AP-1-dependent luciferase activity or relative luciferase units).

**RT-PCR**—Cl41 cells were cultured in a 6-well plate until they reached 85–90% confluence. The cell culture medium was replaced with 0.1% FBS MEM and cultured for 24 h. The cells were then exposed to B[a]PDE or 5-MCDE for 6 h. Cells were washed once with ice-cold PBS and extracted for whole RNA with TRIzol® reagent following the manufacturer's instructions (Invitrogen). The cDNA was synthesized from 1 μg of RNA using the First-Strand Synthesis System for RT-PCR (Invitrogen). The PCR was performed using 2 μl of synthesized cDNA and specific murine VEGF primers (sense: 5′-gcgggctgcctcgcagtc-3′, antisense: 5′-tcaccgccttggcttgtcac-3′) or β-actin primers (sense: 5′-gacctagatggcgcactg-3′, antisense: 5′-gataccacgcttgctctgag-3′). The PCR products were separated on 2% agarose gels. The relative mRNA level of VEGF was normalized to the internal reference β-actin that was coamplified in the same reaction for each sample. RT-PCR was conducted within the linear ranges of PCR cycles and RNA input.

**Western Blot**—Cl41 transfectants were cultured in each well of a 6-well plate to 80–85% confluence with 5% FBS MEM. The cell culture medium was replaced with 0.1% FBS MEM, cultured for 48 h at 37 °C, and then exposed to B[a]PDE or 5-MCDE for various lengths of time. The cells were then washed once with ice-cold PBS and extracted with SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with one of the specific antibodies as indicated. The protein band specifically bound with primary antibodies was detected by using anti-rabbit IgG-AP-linked and ECF Western blotting system.

**EMSA**—Cl41 cells were seeded into 15-cm dishes until 70–80% confluence. The cell culture medium was replaced with 0.1% FBS MEM and cultured for 48 h at 37 °C. The cells were then exposed to 50 μM B[a]PDE for 6 h. Nuclear protein from cells was isolated using the CelLyticTM NuCLEARTM extraction kit (Sigma). Bioinformatics analysis showed that the VEGF promoter region contains three putative AP-1 binding sites. According to those sequences, three EMSA probes were designed and synthesized as shown in Fig. 5a, which were named AP-1.1, AP-1.2, and AP-1.3. The normal wild-type AP-1 consensus sequence was also synthesized as a positive control (AP-1). The oligonucleotides were annealed and 5′-end-labeled with [32P]ATP using standard procedures. A binding reaction was carried out by preincubating 5 μg of nuclear-extracted protein in 20 mM HEPES (pH 7.9), 50 mM NaCl, 5% glycerol,

![FIGURE 3. Effects of PAHs on AP-1 activation.](image-url)
and 0.1 mM dithiothreitol at room temperature for 15 min, which was followed by the addition of double-stranded 32P-labeled oligonucleotides and a second incubation at room temperature for 15 min. For competition assays, a 50-fold excess of unlabeled AP-1 oligonucleotides were added to the binding reaction. Samples were then loaded on a 5% Tris borate/EDTA polyacrylamide gel and electrophoresed for 2 h at 100 V. The gel was dried for 1 h at 80 °C, and radioactivity was detected by autoradiography. Results were subjected to densitometric analysis with Kodak 1D 3.52 for Macintosh, and values were normalized to that obtained in the control sample of each individual probe.

Point Mutation of AP-1 Binding Site in VEGF Promoter Luciferase Reporter—To generate the site-directed mutant of the AP-1 binding sequence in the VEGF promoter region of the VEGF-luciferase reporter plasmid pGL-VEGF, the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer’s instructions. The sense primer was 5′-gctggcgggtaggttaagaatcatcacgcagg-3′ and the antisense primer was 5′-ctcgctggtatgctacacaccgccagcagc-3′. The mutated plasmid was identified and designated as pGL-VEGFmut.

Transient Transfection—1 × 10^6 Cl41 cells were plated into a 10-cm diameter dish and cultured until 80–85% confluence. Transfection was performed using pGL-VEG or pGL-VEGFmut plasmids according to the recommendation of the manufacturer. The cells were then plated into 48-well plates until 80–85% confluence. The transfectants were exposed to B[a]PDE at the indicated dosage for 12 h. The cultures were extracted with 50 μl of lysis buffer, and luciferase activity was measured using the Promega Luciferase assay reagent with a luminometer. The results were expressed as VEGF induction relative to control medium containing 0.1% Me2SO (relative VEGF-luciferase induction).

Statistical Analysis—The Student’s t test was used to determine the significance of the differences of AP-1, HRE activities, or VEGF induction between cells treated with PAHs and medium control alone or various stable transfectants. The differences were considered significant at p ≤ 0.05.

RESULTS

Comparisons of B[a]P, B[a]PDE, CDE, and 5-MCDE in the Effects on VEGF Induction—it has been reported that VEGF is highly expressed in the tumor cells of humans as well as animal models (23–26). To determine whether VEGF is involved in the cell response to PAH exposure, and whether there are differential effects of various PAHs on VEGF induction, Cl41 VEGF mass1 cells were exposed to various PAH compounds. The results showed that B[a]PDE and 5-MCDE markedly induced VEGF transcription, whereas CDE did not show any effect on VEGF expression, and B[a]P only showed a slight inductive effect.
Role of PI-3K/AP-1 Pathway in VEGF Induction by PAHs

fold) at 12 h of exposure (Fig. 1a). To further confirm that B[a]PDE and 5-MCDE can induce the expression of VEGF, RT-PCR was performed. Results were consistent with the VEGF-luciferase reporter assay that B[a]PDE and 5-MCDE increased VEGF transcription (Fig. 1b). Those data indicate that PAHs have differential effects on VEGF induction.

HIF-1α Was Not Involved in VEGF Induction by B[a]PDE and 5-MCDE—It is well known that HIF-1α is an important transcription factor responsible for mediation of VEGF expression under hypoxia conditions (23, 24). In addition, binding sites for HIF-1α in the promoter region of VEGF have been identified (25). To address the potential role of HIF-1α in the regulation of VEGF expression in the cellular response to B[a]PDE and 5-MCDE, HIF-1α response element (HRE)-luciferase reporter was transfected into Cl41 cells, and the stable transfectant, Cl41 HRE mass1, was established. Exposure of the cells to B[a]PDE or 5-MCDE did not induce any HRE transactivation (Fig. 2a). Results from Western blot analysis also showed no HIF-1α protein accumulation in Cl41 cells treated with either B[a]PDE or 5-MCDE (Fig. 2b). These results reveal that the induction of VEGF by B[a]PDE and 5-MCDE might be through an HIF-1α-independent pathway.

AP-1 Transactivation Was Required for VEGF Induction by B[a]PDE and 5-MCDE—Previous studies have shown that AP-1 binding sites in the VEGF promoter region may play a role in the regulation of VEGF expression in some experimental systems (26–28). Our published studies have also demonstrated that exposure of the cells to B[a]PDE and 5-MCDE results in AP-1 transactivation (15, 16). Consistent with our previous findings, treatment of Cl41 cells with either B[a]PDE or 5-MCDE resulted in marked AP-1 transactivation (Fig. 3a), whereas CDE did not show any inductive effect, and B[a]P only showed a marginal effect (Fig. 3b and c). Exposure of cells to B[a]PDE or 5-MCDE also led to marked increases in c-Jun phosphorylation (Fig. 3d), suggesting that c-Jun is a major component involved in B[a]PDE- and 5-MCDE-induced AP-1 activation.

To test whether AP-1 is required for VEGF induction by B[a]PDE and 5-MCDE, a dominant-negative mutant of c-Jun TAM67 was used. After co-transfection, a stable transfectant, Cl41 TAM67 VEGF mass1, was established and identified (Fig. 4a). Overexpression of TAM67 markedly impaired c-Jun phosphorylation in Cl41 TAM67 VEGF mass1 cells compared with that in Cl41 VEGF mass1 cells (Fig. 4b). Inhibition of c-Jun phosphorylation resulted in dramatic blockage of VEGF induction by B[a]PDE and 5-MCDE in both the VEGF-luciferase assay and RT-PCR (Fig. 4, c–e). All these data strongly demonstrate that AP-1 activation is required for VEGF induction by B[a]PDE and 5-MCDE in Cl41 cells.

To further identify whether the regulation of VEGF transcription by AP-1 in the cell response to B[a]PDE exposure is due to its specific binding to the AP-1 binding site, we analyzed the putative AP-1 binding sites in the VEGF promoter region. We found that there are three potential AP-1 binding sites in the VEGF promoter region, including 1242GTCGAAAGTGAGCTATGAGTC (AP-1.1), 1136GTTGATCTGACGCA (AP-1.2), and 943AGTCACTGACCGA (AP-1.3). Thus, EMSA was performed using the AP-1-positive control consensus (GGTTGATCTGACGCAAGAA) (AP-1), and the putative sequences of the VEGF promoter as probes (Fig. 5a). As shown in Fig. 5b, incubation of nuclear extracts from B[a]PDE-treated Cl41 cells with the AP-1 control consensus probe (AP-1) resulted in a marked increase in DNA-protein binding complex formation compared with that of nuclear extracts from cells of medium control (Fig. 5b), suggesting that AP-1 DNA binding activity was induced in cells exposed to B[a]PDE. Interestingly, the B[a]PDE induction of AP-1
FIGURE 6. Effects of Δp85 overexpression on VEGF induction by B[a]PDE and 5-MCDE. CI41 VEGF mass1 cells or CI41 VEGF Δp85 mass1 cells were seeded into each well of a 96-well plate. After culture at 37 °C overnight, the cells were treated with B[a]PDE and 5-MCDE for 12 h at concentrations as indicated (a). For time-course studies (b and c), the cells were treated with 1 μM B[a]PDE (b) or 5-MCDE (c) for the time period as indicated. For dose-response studies (d and e), the cells were treated with different concentrations of B[a]PDE or 5-MCDE for 12 h (d and e). The luciferase activity was measured, and the results are presented as relative VEGF-luciferase induction. The asterisk indicates a significant decrease from CI41 VEGF mass1 treated with either B[a]PDE or 5-MCDE (p < 0.05).
DNAProtein binding complex hand was only observed in the incubation
of nuclear extracts with the AP-1.2 probe, but not with the AP-1.1 or AP-1.3 probes (Fig. 5b). These data strongly indicated that activated AP-1 can specifically recognize and bind to the AP-1.2 binding site within -1136 to -1115 of the VEGF promoter region, whereas putative AP-1 sites in AP-1.1 and AP-1.3 are not directly involved in the regulation of VEGF transcription in cell response to B[a]PDE exposure.

Furthermore, the site-directed mutation of the AP-1.2 site led to a dramatic impairment of VEGF transcription induced by B[a]PDE (Fig. 5c), demonstrating that the AP-1 binding site in AP-1.2 is critical for B[a]PDE-induced VEGF expression. Collectively, these results suggest that AP-1 activated by B[a]PDE can directly bind to the cis-element of the VEGF gene promoter and in turn leads to VEGF expression.

PI-3K Was Required for VEGF Induction by B[a]PDE and 5-MCDE—
It has been well demonstrated that PI-3K activation plays an essential role in AP-1 activation and cell transformation caused by different chemical carcinogens including some PAHs (29–33). Our previous studies indicate that PI-3K activation is a mediator for AP-1 transactivation by B[a]PDE and 5-MCDE in mouse epidermal C141 cells through the Akt/JNK-dependent pathway (15, 16). Jyonouchi et al. (34) also reported that nontoxic concentrations of B[a]PDE could inhibit the differentiation of untransformed human small airway epithelial cells by activating PI-3K (34). Thus, it was of interest to determine whether PI-3K is involved in VEGF induction in cell responses to B[a]PDE and 5-MCDE. C141 VEGF Δp85 mas1, which is a well characterized transfectant in our previous studies (29), was utilized for this investigation. An overexpression of Δp85 can significantly block B[a]PDE- and 5-MCDE-induced PI-3K activation and AP-1 activation (15, 16). Inhibition of PI-3K activation by overexpression of Δp85 dramatically impaired VEGF induction by B[a]PDE and 5-MCDE in all the time points and doses tested (Fig. 6), suggesting that PI-3K activation plays a pivotal role in VEGF induction by B[a]PDE and 5-MCDE. These data, together with the results shown above, indicate that B[a]PDE and 5-MCDE are potent compounds for VEGF induction via PI-3K/AP-1-dependent and HIF-1α-independent pathways.

DISCUSSION

Environmental and occupational exposure to PAHs is associated with increased risk of cancers; however, the molecular mechanisms by which PAHs induce tumors are not yet understood. This study identified for the first time that exposure to PAHs could lead to the increased expression of VEGF in C141 cells. Treatment of C141 cells with B[a]PDE and 5-MCDE resulted in marked induction of VEGF expression, and B[a]P showed some marginal effect, whereas CDE did not show any effect. Further study found that HIF-1α was not activated by the treatment of C141 cells with B[a]PDE and 5-MCDE, whereas AP-1 was significantly activated. Inhibition of AP-1 activation by overexpression of TAM67 resulted in impairment of VEGF induction, suggesting that AP-1 is responsible for VEGF induction by B[a]PDE and 5-MCDE. Results from EMSA and site-directed mutation assays further demonstrated that as a transcription factor, AP-1 recognized and bound to the cis-element of the VEGF promoter and in turn activated VEGF transcription. Further studies indicated that inhibition of PI-3K by overexpression of Δp85 also led to a significant reduction of VEGF expression by B[a]PDE and 5-MCDE. Considering that our previous finding that PI-3K is critical for AP-1 induction by B[a]PDE and 5-MCDE (15, 16), we conclude here that the VEGF induction by B[a]PDE and 5-MCDE is through PI-3K/AP-1-dependent and HIF-1α-independent pathways.

PAHs are ubiquitous environmental pollutants (1, 2). Many of those compounds have been shown to be toxic with carcinogenic potential to humans and cause various cancers in different animal models (6, 7). Exposure of human to PAHs may occur by inhalation, ingestion, and skin contact (36, 37). Non-occupational respiratory exposure is mainly from tobacco smoke and urban air, whereas the major sources of ingested PAHs are from drinking water and cooked food (38, 39). The main route of occupational exposure is, in most industries, inhalation. In many cases, however, skin exposure represents an important route (40–43). Epidemiological studies indicate that populations exposed to carcinogenic PAHs increase in levels of several markers of genotoxicity, including PAH DNA adducts, chromosome aberrations (CA), sister chromatid exchanges (SCE), and ras oncogene overexpression (3, 4). Previous studies have shown that B[a]PDE and 5-MCDE can cause mutation by forming DNA adducts, and initiate tumor formation (7, 8). There is also some evidence that PAHs and their metabolites affect cellular signaling pathways (15–17, 44). Our most recent studies strongly demonstrated that exposure of cells to B[a]PDE and 5-MCDE leads to significant activation of mitogen-activated protein kinases/AP-1, and this activation is mediated by the PI-3K/Akt pathway (15, 16). The present study investigated the possible induction of VEGF in mouse epidermal C141 cells by B[a]P, B[a]PDE, CDE, and 5-MCDE. We found that B[a]PDE and 5-MCDE were most potent compounds for inducing VEGF expression in C141 cells. It was noted that B[a]P had a marginal effect on VEGF induction compared with B[a]PDE. We speculate that part of B[a]P is metabolized into B[a]PDE, which is responsible for VEGF induction. This notion is supported by our previous findings that a slight induction of AP-1 by B[a]P is attributed to its metabolite B[a]PDE (45).

VEGF expression was controlled by several transcriptional factors, such as HIF-1 and AP-1 (51–54). Hypoxia has been thought to be one of the major factors for VEGF induction both in vitro and in vivo (51, 52).

The cellular response to hypoxia is mediated by HIF-1, a heterodimeric protein that can bind to hypoxia response elements (HRE) in the promoter regions of the VEGF gene, and initiates transcription by recruitment of transcriptional co-activators such as CREB/p300 (53). Under normoxic conditions, the α-subunit of HIF-1 is degraded via the ubiquitin-proteasome pathway, preventing formation of active HIF-1. HIF-1α acts as a hypoxia-sensitive switch linking local oxygen concentrations to the stability and activity of the transcription factor, and hence to VEGF expression (53). We found here that B[a]PDE and 5-MCDE are able to induce VEGF expression, whereas they did not show any inductive effect on HIF-1α protein accumulation or HIF-1α-dependent transcriptional activity, indicating that HIF-1α might not be a major mediator for VEGF induction by B[a]PDE and 5-MCDE.

Although VEGF has been well documented as one of the key regulators of angiogenesis (46–48), there is also growing evidence indicating that VEGF is implicated in tumor promotion during the multistep process of chemically induced carcinogenesis (49, 50). Regulation of VEGF has been investigated in chemically induced mouse squamous cell carcinoma of the skin, and the data have shown that VEGF markedly accelerated tumor development (49). Transgenic mice overexpressing VEGF in skin have shown to accelerate papilloma formation in chemical-induced skin carcinogenesis (50). Therefore, VEGF induction by ultimate carcinogenic compounds of B[a]PDE and 5-MCDE might help us understand the molecular mechanisms involved in the carcinogenic effect of those compounds. The VEGF gene promoter region contains AP-1 binding sites, which can be recognized by activated AP-1 transcription factor, so it is proposed that AP-1 may regulate VEGF expression (27, 28, 54). Van Waes and co-workers (55) reported that activation of the MEK/ERK/AP-1 pathway is involved in VEGF expression induced by EGF in human head and neck squamous cell carcinoma.
lines. Shih and Claffey (35) showed that TGF-β potently induces VEGF expression in human HT-1080 fibrosarcomas primarily through both AP-1- and HIF-1-dependent mechanisms. We found here that AP-1 is not only activated in cell response to B[a]PDE and 5-MCDE exposure, but also the inhibition of AP-1 activation by TAM67 overexpression results in a significant impairment of VEGF induction by B[a]PDE and 5-MCDE. The results from EMSA and point mutation analysis showed that B[a]PDE-induced activated AP-1 could directly bind to the AP-1 binding site within −1136 to −1115 of the VEGF promoter region, which is critical for VEGF induction by B[a]PDE. We also demonstrated that PI-3K is essential for B[a]PDE- and 5-MCDE-induced VEGF expression by using Δp85 transfectants. In addition, we found that B[a]PDE and 5-MCDE did not affect HIF-1α activation. So our studies strongly demonstrate that VEGF induction by B[a]PDE and 5-MCDE is specifically mediated by the PI-3K/AP-1-dependent and HIF-1α-independent pathways.

In summary, we have found that B[a]PDE and 5-MCDE exposure leads to VEGF expression in CH1 cells, and this induction is mainly mediated through PI-3K/AP-1-dependent and HIF-1α-independent pathways. Although the detailed molecular mechanisms of initiating signaling pathways by B[a]PDE and 5-MCDE are still under investigation in our laboratory, to some extent, there are many similarities between 5-MCDE and B[a]PDE in VEGF regulation as well as activation of other signaling pathways (15, 16). Elucidation of those similarities, and identification of the signaling initiation mechanisms will not only deepen our understanding of tumor-promoting effects of PAH compounds, but also may provide valuable information for the prevention and therapy of cancers caused by PAHs. This will be one of the major challenges for scientists in this research field.

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