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Exposure to Asphalt Fumes Activates Activator Protein-1 through the Phosphatidylinositol 3-Kinase/Akt Signaling Pathway in Mouse Epidermal Cells

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Millions of tons of asphalt are produced every year and extensively used in the paving and roofing industries (1). It has been estimated that ~2 million workers are exposed to asphalt fumes (1). It was reported that road-paving workers can be exposed to 0.1–2 mg/m³ of bitumen fumes, which can include 10–200 ng/m³ benzo(a)pyrene (2). The worker exposure routes are mainly through inhalation and skin contamination. Prolonged, extensive exposure to asphalt fumes has been reported to be associated with several adverse health effects (3). A major health concern from exposure to asphalt fumes is the potential exposure to carcinogens. Epidemiological studies indicate that there is an increased risk for lung, stomach, nonmelanoma skin cancer, and leukemia in the roofer population (4). Experimental studies using animal and in vitro models demonstrate that laboratory-generated condensates from roofing asphalt fumes are genotoxic and produce skin tumors in mice (5, 6). Exposure to several other asphalt-based paints causes the formation of DNA adducts in the skin and lung of mice as well as in human skin fibroblast (7). However, the information regarding the potential carcinogenic effect of asphalt fumes is very limited.

Chemical carcinogenesis is a complex process that can be divided experimentally into three stages: initiation, promotion, and progression. Initiation is associated with irreversible, carcinogen-mediated DNA mutation. In contrast, promotion is a reversible process in which there are increases in the rate of cell replication and/or alterations in gene expression. Progression represents the final genetic changes associated with the conversion of benign tumors into fully malignant cells. JB6 P+ mouse epidermal cell line (Cl 41), originally derived from primary mouse epidermal cells, offers an excellent model to investigate the molecular events that are associated with tumor promotion. These cells undergo a response analogous to second stage tumor promotion in mouse skin when treated with various tumor promoters. For example, exposure of JB6 P+ cells to 12-O-tetradecanoylphorbol 13-acetate or epidermal growth factor (EGF) induces phenotype of anchorage-independent growth and tumorigenicity in vivo (8–10). These cells have been used extensively as an in vitro model for the promotion of neoplastic transformation (9–15).

AP-1 is a basic leucine zipper transcription factor that is composed of homodimer or heterodimer proteins of the Jun, Fos, or ATF families (14). AP-1 regulates the expression of a diverse array of genes, including those involved in cell growth,

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§ The abbreviations used are: EGF, epidermal growth factor; AP-1, activator protein 1; PI3K, phosphatidylinositol-3-kinase; MAPK, mitogen-activated protein kinase; GSK-3β, glycogen synthase kinase-3β; JNK, c-Jun NH2-terminal kinase; PBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PAH, polycyclic aromatic hydrocarbon; PTEN, tensin homologue deleted on chromosome 10; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PH, Pleckstrin homology; t-JNKI, cell-permeable peptide inhibitor of JNK, dextrorotatory form.
proliferation, and transformation (14–16). It has been demonstrated that the activation of AP-1 is essential for the promotion of skin tumorigenesis (17–21). The activation of AP-1 is regulated by multiple signaling pathways, including phosphatidylinositol-3 kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways. A recent study indicates that AP-1 activity is also regulated by glycogen synthase kinase-3β (GSK-3β) signaling (22). These signal pathways have been shown to play a critical role in promotion of skin tumorigenesis (18, 23–27). The current study was designed to evaluate the effect of exposure to asphalt fumes on AP-1 activity and associated intracellular signaling. Asphalt fumes were generated from asphalt-fume generation systems that simulate road-paving conditions (28). We demonstrate here that exposure to asphalt fumes induces AP-1 transactivation in JB6 P+ cells as well as in skin and primary keratinocytes derived from transgenic mice expressing AP-1 reporter gene; it selectively activates PI3K/Akt and c-Jun NH2-terminal kinase (JNK) pathways. Furthermore, exposure to asphalt fumes promotes anchorage-independent growth in soft agar. These results provide an important insight into the potential role of exposure to asphalt fumes in tumor promotion.

**MATERIALS AND METHODS**

**Materials**—Reagent grade dichloromethane (CH2Cl2, 99.9% pure), hexane, and perdeuterated anthracene were purchased from Aldrich. The PAH mix reference material was purchased from Supelco (Bellefonte, PA). The test asphalt was the type used by the paving industry (Hot Performance Grade Asphalt PG 64-22). The glass-fiber filters (20.3 × 25.4 cm) were purchased from Gelman Sciences (Ann Arbor, Michigan). Solid phase extraction cartridges of EnvirElut PAH (500 ng/2.8 ml) were purchased from Varian (Harpur City, CA). Poly(tetrafluoroethylene) tubes (30 ml) and glass tubes (10 ml) were purchased from Fisher Scientific. Target vias (1.5 mm with 200-μl inserts) were obtained from Alltech Associates, Inc. (Deerfield, IL). Extraction of asphalt fumes from collection media was performed by ultrasonic extraction (FS-220, Ultrasonicator 320W; Fisher Scientific). Extracts were reduced under a nitrogen stream using a TurboVap LV evaporator (Zymark). High purity helium was purchased from Butler Gas Products Co. (McKeees Rocks, PA), and used as gas chromatograph/mass spectrometry (Hewlett Packard, Wilmington, DE) carrier gas. The GC column was HP-5 MS, 95% dimethylpolysiloxane, nonpolar, 30-m length, 0.53 mm inner diameter (Hewlett Packard). All antibodies except anti-actin and anti-p110 subunit of PI3K antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-actin and anti-p110 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Preparation of Asphalt Fume Samples**—Generation of asphalt fumes was conducted in the National Institute for Occupational Safety and Health inhalation facility. A dynamic asphalt fume generation system that simulates road-paving conditions (28). A computer control system has been designed to improve the system’s performance and to simplify its operation. The test asphalt is representative of the type used by the paving industry throughout the midwestern United States. The asphalt fumes were generated at 150 °C, and collected on glass-fiber filters. The samples were prepared for detection, filtration, and preconcentration. In the experiment, the asphalt fumes collected on filter were transferred to a poly(tetrafluoroethylene) tube, and dichloromethane/hexane (50/50) was added. Ultrasonic extraction was performed using an FS-220 Ultrasonicator (320W). After desorption of asphalt fumes from the collection medium, the extract was filtered. Preconcentration was performed under nitrogen using TurboVap LV evaporator. Sample extracts were reconstituted with dichloromethane. The asphalt fumes were generated at 150 °C and used by the paving industry throughout the midwestern United States.

**Ultrasonicator**—The 3-4.5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was employed to determine the number of viable cells in culture (Boehringer Mannheim) (32). The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. Briefly, the cells were plated into 96-well microtiter plates and exposed to either asphalt fumes or control fumes (10 μg/ml or 100 μg/ml). After 1 h of incubation, the MTT labeling reagent was added to each well, and the plates were incubated at 37 °C for 4 h. The cultures were then solubilized, and spectrophotometric absorbance of the samples was detected by a microtiter plate reader. The wavelength to measure absorbance of formazan product is 570 nm, with a reference wavelength of 750 nm.

**PI3K Activity**—Cells were washed with ice-cold phosphate-buffered saline, scraped from the plates, and centrifuged at 4000 rpm for 5 min. The cell pellet was incubated for 30 min on ice in lysis buffer (150 mM NaCl, 0.1% Triton X-100, 10 mM NaF) supplemented with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 2 mM leupeptin, and 2 mM aprotinin, and centrifuged at 15,000 × g for 15 min. PI3K was immunoprecipitated using a method described previously (33). Briefly, 400 μg of total protein was incubated with 20 μl of protein A/G plus agarose beads (24 μg of protein A/G plus agarose beads was added for each 10 μg of protein), followed by aspiration of beads. The supernatant was then incubated with 10 μl of antibody directed against p110 subunit of PI3K (Santa Cruz Biotechnology) overnight at 4 °C. Protein A/G-agarose beads (30 μl) were added for an additional 1 h. The beads were then pelleted and washed sequentially, five times, with 20 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM EDTA and once with 20 mM Hepes, pH 7.5. PI3K activity assay was performed using 7.5% SDS polyacrylamide gel electrophoresis (PAGE) and dried gel as substrate in a final volume of 50 μl containing 20 μl HEPEs, pH 7.5, 10 mM MgCl2, 2 μM of [γ-32P]ATP, 60 μM ATP, and 0.2 mg/ml sonicated phosphatidylinositol. Reaction was carried out for 15 min at room temperature and extracted by the addition of 80 μl of 1 N HCl and 2.5 N NaOH.
160 μl of chloroform/methanol (1:1). After centrifugation at 3000 cpm for 2 min, the organic phase (bottom layer) was collected and dried in with a vacuum drier (SpeedVac, Thermo Savant, Holbrook, NY). The samples were dissolved in 10 μl of chloroform and separated on a thin layer chromatography plate. Incorporation of 32P into phosphorylated lipids was detected by autoradiography for 1–2 days.

**Immunoblotting**—The immunoblotting procedure for detecting phosphorylation and expression of signal proteins was performed as described previously (33). Briefly, cells were washed with phosphate-buffered saline and lysed with radioimmunoprecipitation assay buffer for 10 min, solubilized cells were centrifuged, the supernatant was collected, and the protein concentration was determined. Aliquots of the protein (40 μg) were loaded onto the lanes of an SDS 10.0% polyacrylamide gel. The proteins were separated by electrophoresis, and the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5% nonfat dry milk or 5% bovine serum albumin (for detection of phosphorylation) in 0.010M phosphate-buffered saline, pH 7.4, and 0.05% Tween 20 at room temperature for 1 h to block nonspecific immunoreactivity. Subsequently, the membranes were incubated with primary antibodies directed against signal proteins for 1.5 h at room temperature. After two quick washes in phosphate-buffered saline and 0.05% Tween 20, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences) diluted at 1:2000 in phosphate-buffered saline and 0.05% Tween 20 for 1 h. The immune complexes were detected by the enhanced chemiluminescence method (Amersham Biosciences). In some cases, the blots were stripped and re-probed with an anti-actin antibody (Santa Cruz Biotechnology).

**Anchorage-independent Growth**—The cell growth matrix consists of two layers of basal medium Eagle agar in 6-well culture trays. The base layer (2 ml) contained 10% FBS and 0.5% basal medium Eagle agar. The top layer (0.5 ml) contained 10% FBS, 0.53% basal medium Eagle agar, and J6E P- or J6E*P*P* cell suspension (0.5 × 10⁶). EGF (30 ng/ml) and asphalt fumes (30 ng/ml) were applied in both the top and bottom layers. The cultures were maintained at 37 °C with an atmosphere of 5% CO₂ for 14 days, and the number of induced cell colonies was counted under a microscope. Colonies, which contain 8 or more cells, were counted in four 0.5-cm² areas randomly chosen with respect to distance from the center of the well, and the count was multiplied by the appropriate factor to give the colony number/well.

**Statistical Analysis**—Differences among treatment groups were tested using an analysis of variance. Differences in which p was less than 0.05 were considered statistically significant. In cases where significant differences were detected, specific post hoc comparisons between treatment groups were examined with Student-Newman-Keuls tests.

**RESULTS**

**Determination of Asphalt Fume Concentration**—The concentration of asphalt fumes was determined by gas chromatography/mass spectrometry with positive electron ionization. The instrument was calibrated with the use of a mixture of 16 reference PAHs, and perdeuterium anthracene was used as internal standard. The total ion chromatograms were acquired with a 3-min solvent delay. Separation was performed on a HP-5 MSD capillary column (30-m length, 0.53 mm inner diameter) with a temperature program from 50–310 °C at an increasing rate of 5 °C/min. Calibration curves were developed with five-point measurements. The recovery of the asphalt fumes were evaluated by adding stable isotope perdeuterated anthracene in the samples and determined by gas chromatography/mass spectrometry. The analytical results were recorded as profiles of total ion chromatogram over specific ranges of mass-to-charge ratios. A typical total ion chromatogram acquired is displayed in Fig. 1A. The major components were observed over a range of molecular size of m/z 51–365 (Fig. 1B and C), and eluted from capillary column at retention times of 13–52 min. The relative recovery of the internal standard can account for losses of the analytes during sample preparation and detection. The total asphalt fume concentration was determined and diluted to 41.41 mg/ml with Me₂SO for cell exposi-
Exposure to Asphalt Fumes Activates AP-1

The activity of AP-1 is regulated by multiple signaling pathways. Among these pathways, PI3K- and MAPK-mediated signaling plays a critical role. Therefore, we sought to determine whether asphalt fumes affected PI3K and MAPKs. As shown in Fig. 4A, exposure to asphalt fumes activated PI3K, and maximal activation occurred at 1–2 h after exposure. However, asphalt fumes did not affect the phosphorylation and expression of phosphatase and tensin homologue deleted on chromosome-10 (PTEN), a dual-specificity phosphatase that dephosphorylates PtdIns(3,4,5)P3 (Fig. 4B), suggesting that the effect of asphalt fumes is not mediated by the inhibition of dephosphorylation. One major substrate of PI3K is PKB/Akt. Activated PI3K stimulates phosphoinositide-dependent kinase-1/2, which in turn phosphorylates Akt on Thr-308 and Ser-473 (34, 35). As shown in Fig. 5A, exposure to asphalt fumes induced phosphorylation of Akt at Ser-473 and Thr-308. We further investigated whether the activation of Akt is mediated by PI3K. Blockage of PI3K by a specific inhibitor, LY294002, eliminated asphalt fume-mediated phosphorylation of Akt (Fig. 5B). A similar blocking effect was observed when wortmannin, another PI3K inhibitor, was applied (data not shown). In addition, inhibition of PI3K activation by expressing dominant-negative p85 (regulatory subunit of PI3K) also blocked asphalt fume-induced Akt activation (Fig. 5C). To determine whether different solvents may affect biological activity of asphalt fumes, we dissolved asphalt fumes with ethanol. Effect of ethanol-dissolved asphalt fumes on signal transduction was evaluated, and the results were similar to those of Me2SO-dissolved asphalt fumes (data not shown).

Next, we sought to determine whether exposure to asphalt fumes activated downstream components of Akt. Three major downstream effectors of Akt (GSK-3β, p70 S6 kinase, and forkhead transcription factor) were examined. Exposure to asphalt fumes induced phosphorylation of GSK-3β on Ser-9 (Fig. 6A) without affecting phosphorylation on Tyr-216 (data not shown). The antibody directed against phospho-p70 S6 kinase (Thr-421/Ser-424; Cell Signaling Inc.) also reacted with phospho-p85 S6 kinase. Exposure to asphalt fumes increased phosphorylation of p85 S6 kinase and p70 S6 kinase on Thr-421/Ser-424 (Fig. 6A), but not on Thr-389 (data not shown). However, exposure to asphalt fumes did not affect the phosphorylation of either p38 MAPK or Erk1/2, which lasted for only 15 min, and modestly enhanced phosphorylation of c-Jun. In contrast, asphalt fumes did not affect the phosphorylation of either p38 MAPK or ERKs.

PI3K/Akt Signaling Pathway Mediates Asphalt Fume-stimulated AP-1 Activity and Anchorage Independence—To determine which signaling pathway was involved in asphalt fumes-stimulated AP-1 activation, we used specific inhibitors to block the activity of kinases that may regulate AP-1 activity. As shown in Fig. 8, treatment with the PI3K inhibitor LY294002 completely eliminated asphalt fume-stimulated AP-1 activity. Similarly, another PI3K inhibitor, wortmannin, also blocked asphalt fume-induced AP-1 activation (data not shown). 4-Benzyl-2-methyl-1,2,4-thiazolidine-3,5-dione is a specific GSK-3β inhibitor (36). As shown in Fig. 8, 4-benzyl-2-methyl-1,2,4-thiazolidine-3,5-dione partially but significantly blocked AP-1 activity. n-JNKI is a specific JNK inhibitor, and we have shown...
previously that it eliminates JNK activity (37). D-JNKI had little effect on asphalt fume-mediated AP-1 activation. The effect of other MAPK inhibitors was also examined. Neither SB202190 (inhibitor for p38 MAPK) nor PD98059 (inhibitor for MEK1) altered asphalt fume-regulated AP-1 activity (data not shown).

Effect of asphalt fumes on transformation of JB6 P⁺/H11001 cells was examined by assaying anchorage-independent growth. It has been shown that tumor promoter 12-O-tetradecanoylphorbol 13-acetate and epidermal growth factor (EGF) promote anchorage-independence of JB6 P⁺ cells (23). As shown in Fig. 9, asphalt fumes promoted basal as well as EGF-mediated anchorage-independent growth. However, in JB6 P⁺ cells constitutively expressing dominant-negative p85 (JB6 ΔIno⁺/Δ5), asphalt fumes were ineffective. Thus, PI3K was an essential component for asphalt fume-promoted JB6 P⁺ cell transformation.

DISCUSSION

Asphalt is an extremely complex and variable mixture. It contains aliphatic PAHs, heterocyclic compounds, and some nitrogen-, oxygen-, and sulfur-containing compounds (5). These persistent organic compounds are very nonpolar and exhibit a high accumulation potential in living systems (38). The highly lipophilic PAH chemicals constitute an extraordinarily large and diverse class of organic molecules and represent components with a wide range of molecular sizes and structural types. It has been estimated that that crude asphalt contains the most widely distributed class of potent carcinogens present in the human environment (39). To evaluate adverse biological

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**FIG. 3. Effects of asphalt fumes on AP-1 activity.** A, AP-1 activity in JB6 P⁺ cells. Cells were exposed to asphalt fumes (AF, 0 or 20 μg/ml) for 6–48 h. AP-1 activity was determined as described under “Materials and Methods.” Each data point is the mean of four replicates ± S.E. (bars). B, AP-1 activity in transgenic mice expressing AP-1 reporter gene. Skin of transgenic mice was painted with asphalt fumes for 24 h. Equal areas of skin (2 mm²) were removed before and after asphalt fume painting and assayed for AP-1 activity. Purified keratinocytes from transgenic mice were cultured as described under “Materials and Methods” and exposed to asphalt fumes (10 μg/ml, 6 h). AP-1 activity in keratinocytes was determined as described above. Each data point is the mean of four independent trials ± S.E. (bars). *, p < 0.05, statistically significant difference between control and asphalt fume-treated samples.

**FIG. 4. Effect of asphalt fumes on PI3K activity in JB6 P⁺ cells.** A, PI3K activity. JB6 P⁺ cells were grown in serum-free medium for 24 h and exposed to asphalt fumes (AF, 20 μg/ml). PI3K was immunoprecipitated and the kinase activity was measured in the immunocomplexes using phosphatidylinositol and [γ³²P]ATP as substrates. The products were separated by thin layer chromatography and subjected to autoradiography as described under “Materials and Methods.” B, phosphorylation of PTEN. The phosphorylation of PTEN on Ser-380 was determined with immunoblot using a phosphospecific antibody. The same blot was stripped and reprobed with either an anti-PTEN or an anti-actin antibody. The experiments were replicated three times.
effects of asphalt fumes in occupational exposure, we have established a computer-controlled dynamic asphalt fume generation system in which asphalt fumes can be generated under simulated road-paving conditions (28). We have also developed a highly sensitive, selective, and reliable analytical method to characterize the contents of asphalt fumes generated under these conditions (28). Human skin receives much occupational exposure to asphalt fumes. We are therefore interested in examining the effect of exposure to asphalt fumes on epidermal cells.

Exposure to asphalt fumes activates AP-1 in a mouse epidermal cell line as well as in the skin and primary keratinocytes derived from transgenic mice. Furthermore, asphalt fumes promote cell transformation. Up-regulation of AP-1 activity is frequently associated with cell transformation, and blockage of AP-1 activity has been shown to reverse the transformation of mouse epidermal cells (10, 13, 17, 18, 40). Many intracellular signaling pathways either directly or indirectly regulate AP-1 activity and are involved in skin tumorigenesis (14, 24, 27). However, they are minimally involved in asphalt fume-induced AP-1 activation. Although JNKs are modestly and transiently activated by asphalt fumes, the extent of activation apparently does not lead to AP-1 activation; blockage of JNK activation has little effect on asphalt fume-induced AP-1 activation. Asphalt fumes are ineffective in the activation of p38 MAPK and ERKs, indicating that the effect of asphalt fumes on intracellular signaling is somewhat specific. As expected, blockage of either p38 MAPK or ERKs does not affect asphalt fume-mediated AP-1 activation.

PI3K is an important signaling molecule consists of a heterodimer of a 110-kDa (p110) catalytic subunit and an 85-kDa (p85) regulatory subunit. Upon activation, which is usually triggered by the association with tyrosine kinases or G-protein-coupled receptors, it phosphorylates phosphatidylinositol (PtdIns) and generated phosphorylated derivative (phosphoinositide), such as PtdIns(3) phosphate, PtdIns(3,4)P2, PtdIns(3,4,5)P3 (41). Phosphoinositides interact with the Pleckstrin homology (PH) motif and subsequently activate PH domain containing proteins. Akt is among the first protein known to contain a PH domain. PH domain of Akt specifically binds to PtdIns(3) phosphate, PtdIns(3,4)P2 and PtdIns(3,4,5)P3. Upon activation, Akt contains substantial levels of PtdIns(3) phosphate, but hardly any PtdIns(3,4)P2 and PtdIns(3,4,5)P3. Upon activation of PI3K, PtdIns(3,4)P2 and PtdIns(3,4,5)P3 are synthesized at plasma membrane and interact with Akt through its PH domain. This induces (1) the translocation of Akt to the plasma membrane and (2) a conformational change that exposes Thr-308 (kinase domain) and Ser-474 (C-terminal regulatory domain) phosphorylation sites at Akt. The phosphorylation of Akt was then catalyzed by PDK1 at plasma membrane (41). Phosphorylation of both these residues is essential for maximal activation of Akt in response to PI3K activation. Our result indicates that asphalt fumes enhance PI3K activity and stimulate phosphorylation of Akt on both Thr-308 and Ser-474 without affecting the expression and phosphorylation of PTEN. PTEN antagonizes the action of PI3K activity by dephosphorylating PtdIns(3,4,5)P3 (42). Thus, asphalt fume-induced Akt
activation is less likely to be mediated by an inhibition of PTEN. A number of studies have documented PI3K-independent activation of Akt (41). Our study clearly demonstrates that asphalt fume-induced Akt activation is PI3K-dependent; blockage of PI3K activity abolishes asphalt fume-induced Akt phosphorylation.

The mitogen-stimulated protein kinase p70S6K/p85S6K is a Ser/Thr kinase that plays an essential role in cell proliferation and growth. p70S6K phosphorylates the 40 S ribosomal protein S6 and is involved in translational control of 5'-oligopyrimidine tract mRNAs (43, 44). p85S6K, an isoform of p70S6K, is derived from the same gene and is identical to p70S6K except for 23 extra residues at the N terminus that encode a nuclear localizing signal (43). The activation of p70S6K can be PI3K/Akt-dependent or -independent (45). PI3K/Akt-dependent activation is mediated by mammalian target of rapamycin. Asphalt fumes apparently promote phosphorylation of p70S6K/p85S6K in a PI3K/Akt-dependent manner; blockage of PI3K eliminates

Fig. 6. Effect of asphalt fumes on Akt-regulated signaling in JB6 P+ cells. A, phosphorylation of downstream signal components of Akt. Phosphorylation of p70/p85 S6 kinase (Thr-421/Ser-424), GSK-3β (Ser-9), and forkhead transcription factor (FKHR) (Ser-256) was determined with immunoblots using phosphospecific antibodies. The same blots were stripped and probed with an anti-actin antibody. Effect of LY294002 (LY) on asphalt fume-induced activation of p70/p85 S6 kinase (B) and GSK-3β (C). JB6 P+ cells were pretreated with LY294002 (0 or 10 μM) for 30 min and exposed to asphalt fumes. Phosphorylation of p70 S6 kinase and GSK-3β was determined as described above. The experiments were replicated three to four times.

Fig. 7. Effects of asphalt fumes on the activation MAP kinases in JB6 P+ cells. Phosphorylation of JNKs, p38 MAPK, and ERKs was investigated with immunoblots using phosphospecific antibodies. The same blots were stripped and probed with an anti-actin antibody. The experiments were replicated three to four times.

Fig. 8. Effect of inhibitors for PI3K, JNKs, and GSK-3β on asphalt fume-induced AP-1 activation in JB6 P+ cells. JB6 P+ cells were pretreated with 10 μM LY294002 (LY; PI3K inhibitor), 1 μM D-JNKI (JNKi; JNK inhibitor), and 10 μM 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (GSKi; GSK-3β inhibitor) 30 min before AF exposure. AP-1 activity was assayed 12 h after exposure to asphalt fumes. The experiment was replicated four times. *, p < 0.05, statistically significant difference from untreated controls; **, p < 0.05, statistically significant difference from paired, asphalt fume-exposed groups. Ct, control; DMSO, Me2SO.
Asphalt Fumes Activate AP-1 and Intracellular Signaling

Fig. 9. Effect of asphalt fumes on anchorage-independent growth of JB6 P* cells. JB6 P* cells or JB6DNp85 cells, which were grown in a matrix of soft agar, were exposed to AF (0 or 20 ng/ml) plus EGF (0 or 20 ng/ml). Cell colonies were scored after 14 days of incubation at 37 °C in an atmosphere of 5% CO2. The number of colonies was expressed as an arbitrary unit relative to the untreated control group. **, p < 0.05, statistically significant difference from untreated controls. *, p < 0.05, statistically significant difference from paired, EGF-treated groups.

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