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Occupational exposure to asphalt fumes may pose a health risk. Experimental studies using animal and *in vitro* models indicate that condensates from asphalt fumes are genotoxic and can promote skin tumorigenesis. Enhanced activity of activator protein-1 (AP-1) is frequently associated with the promotion of skin tumorigenesis. The current study investigated the effect of exposure to asphalt fumes on AP-1 activation in mouse JB6 P^+ epidermal cells and the skin of transgenic mice expressing the AP-1 luciferase reporter gene. Asphalt fumes were generated from a dynamic generation system that simulated road-paving conditions. Exposure to asphalt fumes significantly increased AP-1 activity in JB6 P^+ cells as well as in cultured keratinocytes isolated from transgenic mice expressing AP-1 reporter. In addition, topical application of asphalt fumes by painting the tail skin of mice increased AP-1 activity by 14-fold. Exposure to asphalt fumes promoted basal as well as epidermal growth factor-stimulated anchorage-independent growth of JB6 P^+ cells in soft agar. It activated phosphatidylinositol 3-kinase and induced phosphorylation of Akt at Ser-473/Thr-308, and concurrently activated downstream p70 S6 kinase as well as glycogen synthase kinase-3β. Asphalt fumes transiently activated c-Jun NH_2-terminal kinases without affecting extracellular signal-regulated kinases and p38 mitogen-activated protein kinases. Further study indicated that blockage of phosphatidylinositol 3-kinase activation eliminated asphalt fume-stimulated AP-1 activation and formation of anchorage-independent colonies in soft agar. This is the first report showing that exposure to asphalt fumes can activate AP-1 and intracellular signaling that may promote skin tumorigenesis, thus providing important evidence on the potential involvement of exposure to asphalt fumes in skin carcinogenesis.

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/m^3 of bitumen fumes, which can include 10–200 ng/m^3 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)[1] 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 (9–13).

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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[1] 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 NH_2-terminal kinase; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; PAH, polycyclic aromatic hydrocarbon; PTEN, tensin homologue deleted on chromosome-10; PtdIns, phosphatidyl inositol; $PtdInsP_2$, phosphatidylinositol biphosphate; $PtdInsP_3$, phosphatidylinositol triphosphate; PH, Pleckstrin homology; D-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 a dynamic asphalt-fume generation system that simulates 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 NH_2-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 (CH_2Cl_2, 99.9+%), hexane, and perdeuterated anthracene were purchased from Aldrich. The PAH mix reference material was purchased from Supelo (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 (Harbor City, CA). Poly(tetrafluoroethylene tubes (30 ml) and glass tubes (10 ml) were purchased from Fisher Scientific. Target vials (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. (Mckees Rocks, PA), and used as gas chromatography/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 was employed to provide test asphalt fumes (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 fumes sample preparation involved desorption, 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.

Culture and Treatment of Mouse Epidermal Cell Lines—JB6 P^+ mouse epidermal cell line (Cl 41) were grown in Eagle's minimal essential medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 μg/ml gentamicin at 37 °C with 5% CO_2. The stable transfectants of Cl 41 cells expressing AP-1-luciferase reporter (Cl 41 AP-1) and dominant-negative PI3K regulatory p85 subunit ($JB6^{DNp85}$) have been described previously (24). Cells were exposed to asphalt fumes (10–320 μg/ml) for specified periods. For blocking intracellular kinase activity, cells were treated with either PI3K inhibitor (10 μM LY294002 or 100 nM wortmannin; Sigma), GSK-3β inhibitor (10 μM 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; Calbiochem), or JNK inhibitor (1 μM D-JNKI; Alexis Biochemicals, San Diego, CA) 30 min before exposure to asphalt fumes.

AP-1-luciferase Reporter Transgenic Mice and Keratinocyte Culture—2X TRE-luciferase reporter transgenic mice were originally established by Rincon and Flavell (29). A C57BL/6 male mouse carrying the 2X TRE-luciferase transgene was crossed with DBA/2 female mice (SASCO, Omaha, NE) (18). The F1 offspring were screened by testing both the basal and 12-*O*-tetradecanoylphorbol 13-acetate-induced levels of luciferase activity for the presence of the AP-1-luciferase reporter gene. Mice were housed under temperature-, humidity-, and light-controlled conditiona. Food and water were available *ad libitum*.

Primary mouse epidermal keratinocytes were isolated from the epidermis of newborn AP-1 transgenic mice by a method described previously (30, 31). Briefly, the trunk skin of newborn mice was floated dermis side down on 0.25% trypsin/EDTA in Dulbecco's phosphate-buffered saline solution overnight at 4 °C, and the epidermal layer was separated. The epidermis was minced in keratinocyte essential medium (Sigma), and then the cell suspension was filtered through a 70-μm cell strainer to remove the stratum corneum. Keratinocytes were collected by centrifugation (500 × *g*, 10 min) and seeded into fibronectin/collagen-coated dishes. Cells were maintained in a keratinocyte growth medium (Sigma) for 4 h with 5% CO_2 at 37 °C. Unattached cells were removed by a gentle wash with warm keratinocyte growth medium. Adhesive keratinocytes were cultured in this medium at 37 °C in a humidified atmosphere of 5% CO_2.

Measurement of AP-1 Activity—AP-1 transactivation in JB6 P^+ epidermal cells was determined by assaying the activity of the luciferase reporter (24). Briefly, cells were cultured in 96-well plates and grown in a medium containing 10% FBS. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2. For assaying AP-1 activity, subconfluent cultures were maintained in a medium containing 0.1% FBS for 24 h and subsequently treated with asphalt fumes with or without protein kinase inhibitors for specified durations. After treatment, cellular protein was extracted with a 1× lysis buffer supplied in the luciferase assay kit (Promega), and luciferase activity was measured with a Monolight luminometer (Analytical Luminescence Laboratory, Sparks, MD). AP-1 activity (luciferase activity) was calculated and expressed relative to the untreated cultures. AP-1 activity was also measured in purified primary keratinocytes and the skin of transgenic mice expressing AP-1 luciferase reporter gene. Primary epidermal keratinocytes were exposed to asphalt fumes (10 μg) for 6 h, and AP-1 activity was determined. For assay of AP-1 activity in mouse skin, a small piece of tail skin (2 mm^2) was removed either before or after asphalt painting, and cellular protein was extracted with a 2× lysis buffer, then luciferase activity was assayed as above.

MTT Assay—The 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was employed to determine the number of viable cells in culture (Roche Molecular Biochemicals) (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 (10–320 μg/ml) or Me_2SO (control) for 48 h. After exposure to asphalt fumes, 10 μl of MTT labeling reagent were 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 cpm for 5 min. The cell pellet was incubated for 30 min on ice in lysis buffer (150 mM NaCl, 100 mM Tris-HCl, pH 8.0, 1% Triton X-100, 5 mM EDTA, and 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 for 1 h at 4 °C, followed by spinning at 3000 cpm for 3 min. 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. PI3K activity assay was performed using phosphatidylinositol as substrate in a final volume of 50 μl containing 20 mM HEPES, pH 7.5, 10 mM MgCl, 2 μCi of [γ^{32}P]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 M HCl and

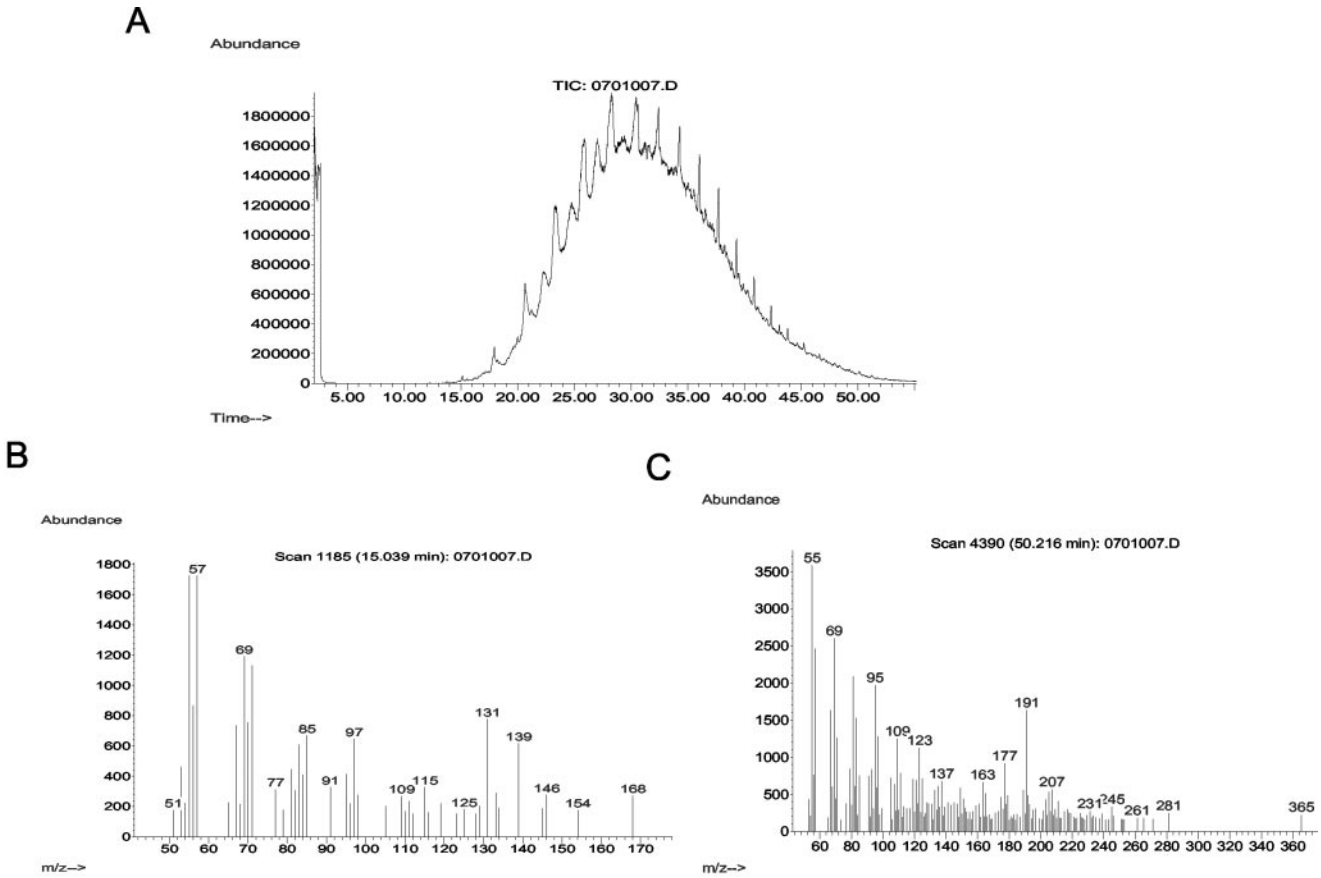

FIG. 1. **Determination of asphalt fumes concentration.** *A*, total ion chromatogram of asphalt fumes collected on glass-fiber filters acquired by positive electron ionization gas chromatography/mass spectrometry. *B*, mass spectrum corresponding to retention time of 15.0 min. *C*, mass spectrum corresponding to retention time of 50.2 min.

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 ^{32}P 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 0.010 M 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, 0.33% basal medium Eagle agar, and JB6 P^+ or $JB6^{DNp85}$ cell suspension (0.5×10^4). EGF (30 ng/ml) and asphalt fumes (10 ng/ml) were applied in both the top and bottom layers. The cultures were maintained at 37 °C with an atmosphere of 5% CO_2 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-cm^2 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. 1*A*. The major components were observed over a range of molecular size of *m/z* 51–365 (Fig. 1, *B* 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 Me_2SO for cell expo-

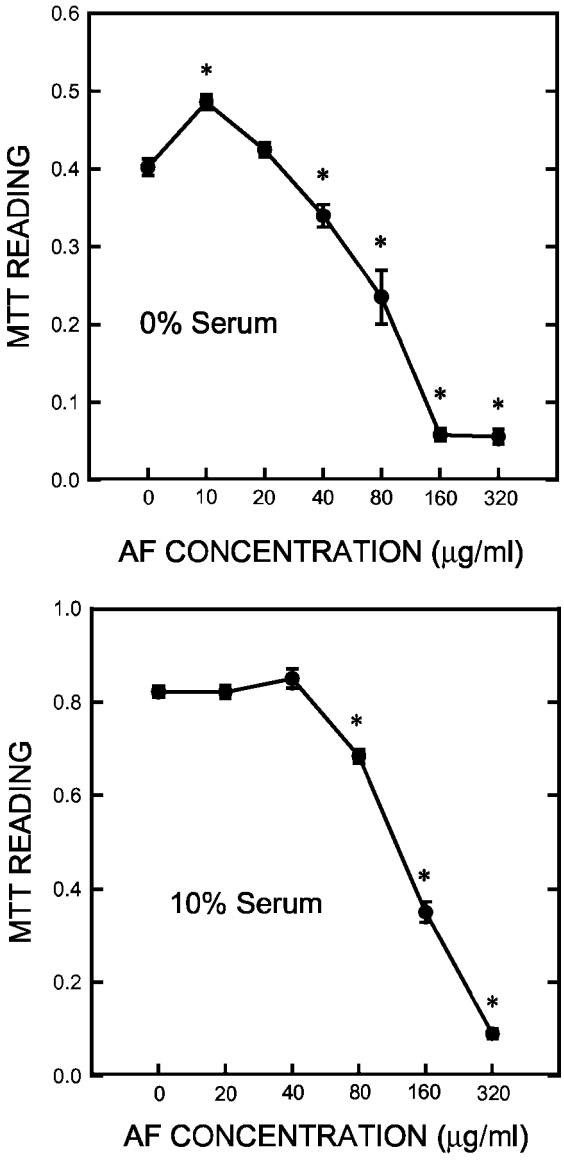

FIG. 2. **Effect of asphalt fumes on the viability of JB6 P$^+$ cells.** Cells were grown in either serum-free medium or medium containing 10% serum and exposed to asphalt fumes (*AF*; 0–320 μg/ml) dissolved in Me_2SO for 48 h. The controls were exposed to Me_2SO. The viability of cells was determined by MTT assay as described under "Materials and Methods." Each *data point* is the mean of five replicates ± S.E. (*bars*). *, $p < 0.05$, statistically significant difference between control and asphalt fume-treated cells.

sure. In some cases, asphalt fumes were dissolved in ethanol for comparison.

Exposure to Asphalt Fumes Activates AP-1—MTT assay was performed to determine whether asphalt fumes, at a pharmacologically relevant concentration range, affect cell viability. As shown in Fig. 2, at the concentration less than 40 μg/ml, asphalt fumes did not significantly affect the viability of JB6 P$^+$ cells; however, at high concentrations (>40 μg/ml), asphalt fumes caused cell death regardless of serum concentration in the culture medium. In all the subsequent studies, therefore, a non-toxic concentration of asphalt fumes (20 μg/ml) was used. Exposure to asphalt fumes significantly stimulated AP-1 activity in JB6 P$^+$ cells, and maximal activation occurred at 12 to 24 h after treatment with asphalt fumes (Fig. 3*A*). Furthermore, exposure to asphalt fumes dramatically increased AP-1 activity in the skin and purified keratinocytes of transgenic mice expressing AP-1 reporter gene (Fig. 3*B*).

Exposure to Asphalt Fumes Activates PI3K/AKT Signaling Pathway—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. 4*A*, 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)P_3 (Fig. 4*B*), 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. 5*A*, 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. 5*B*). 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. 5*C*). 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 Me_2SO-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. 6*A*) 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. 6*A*), but not on Thr-389 (data not shown). However, exposure to asphalt fumes did not affect the phosphorylation of forkhead transcription factor, a distal component of PI3K/Akt signaling pathway. Blockage of PI3K activity by LY294002 eliminated asphalt fume-mediated phosphorylation of p85 S6 kinase and p70 S6 kinase, and partially inhibited asphalt fume-induced GSK-3β (Fig. 6, *B* and *C*).

The effect of exposure to asphalt fumes on MAPK was examined. As shown in Fig. 7, asphalt fumes induced a transient activation of JNKs, 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-thiadiazolidine-3,5-dione is a specific GSK-3β inhibitor (36). As shown in Fig. 8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione partially but significantly blocked AP-1 activity. D-JNKI is a specific JNK inhibitor, and we have shown

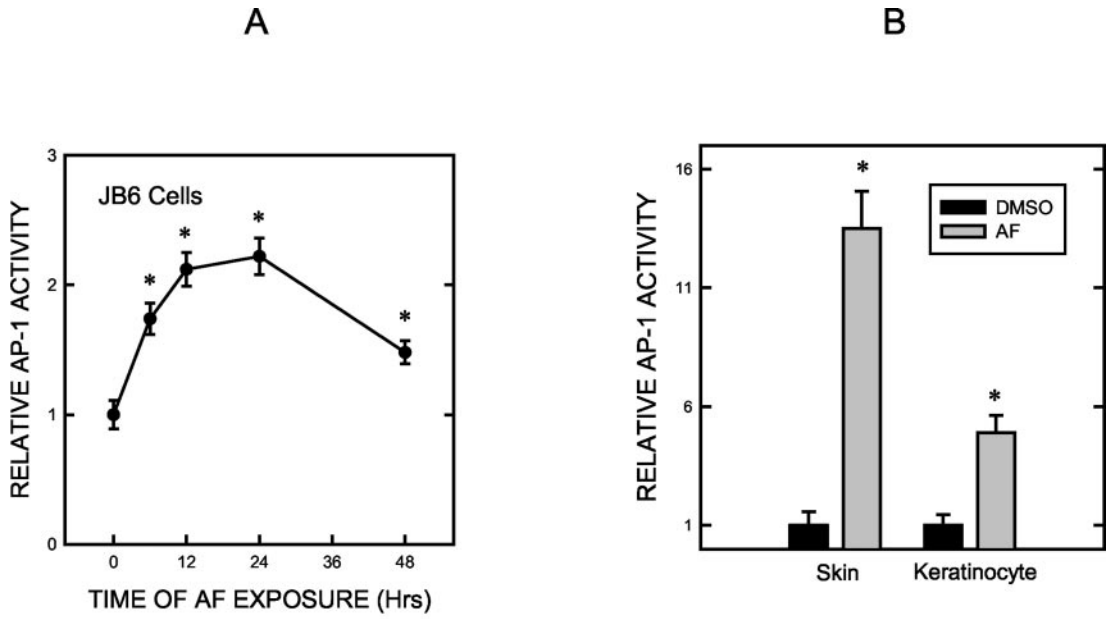

FIG. 3. **Effects of asphalt fumes on AP-1 activity.** *A*, AP-1 activity in JB6 P^+ cells. Cells were exposed to asphalt fumes (*AF*, 0 or 20 μg/ml) for 6–48 h. AP-1 activity was determined as described under "Materials and Methods." Each *data point* is the mean of four replicates ± S.E. (*bars*). *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 mm^2) 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.

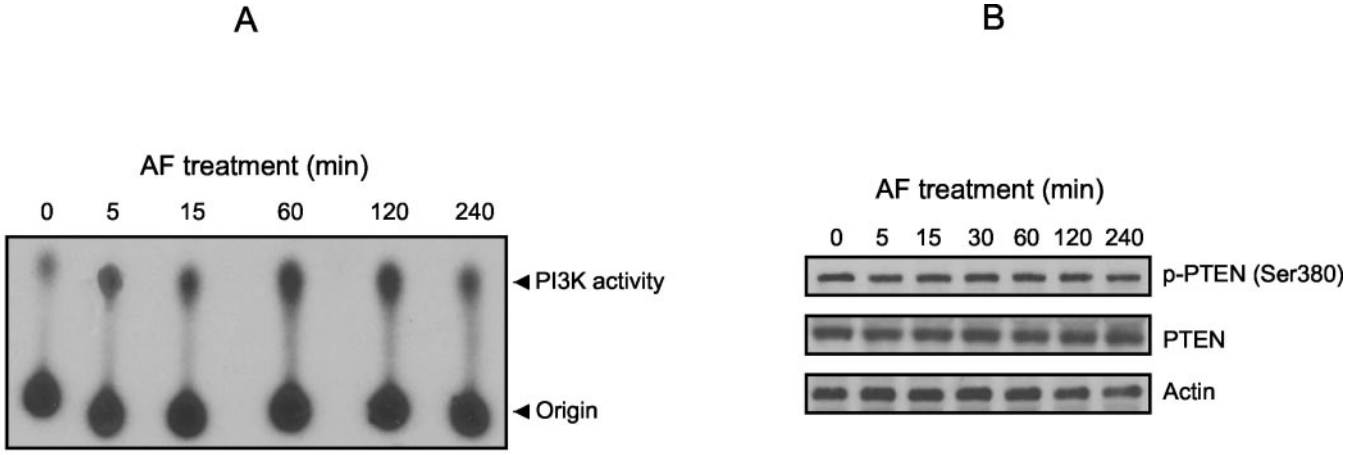

FIG. 4. **Effect of asphalt fumes on PI3K activity in JB6 P^+ cells.** *A*, PI3K activity. JB6 P^+ cells were grown in serum-free medium for 24 h and exposed to asphalt fumes (AF, 20 μg/ml). PI3K was immunoprecipitated and the kinase activity was measured in the immunocomplexes using phosphatidylinositol and [γ^{32}P]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 striped and reprobed with either an anti-PTEN or an anti-actin antibody. The experiments were replicated three times.

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^+ 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 ($JB6^{DNp85}$), 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

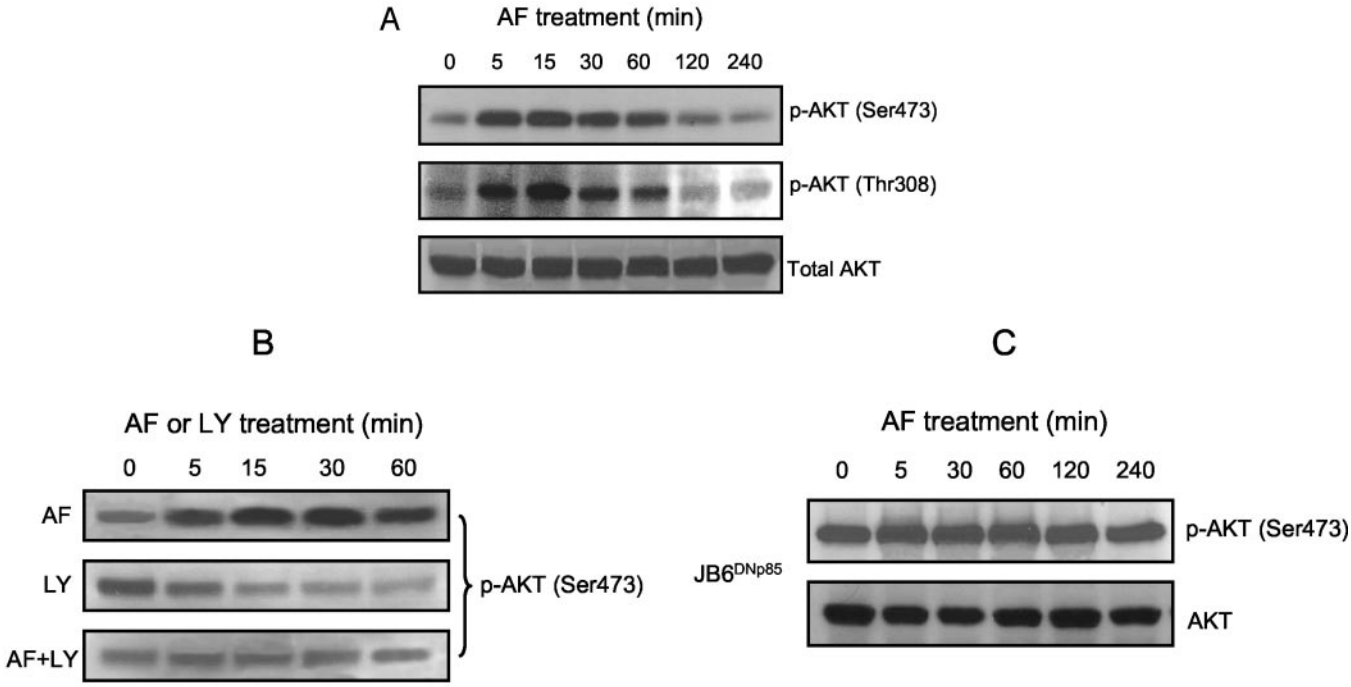

FIG. 5. **Effects of asphalt fumes on Akt activation in JB6 P^+ and JB6^{DNp85} cells.** *A*, Akt phosphorylation. Cells were grown in a serum-free medium for 24 h. After serum starvation, cells were treated with asphalt fumes (*AF*, 20 μg/ml) for various times (5–120 min). Phosphorylation (Ser-473 and Thr-308) and expression of Akt were determined with immunoblot using specific antibodies against either phosphorylated or regular forms of Akt. *B*, effect of LY294002 on asphalt fume-induced Akt activation. JB6 P^+ cells were pretreated with LY294002 (0 or 10 μM) for 30 min and exposed to asphalt fumes. Phosphorylation of Akt was determined with immunoblot as described above. *C*, phosphorylation and expression of Akt in JB6^{DNp85} cells. The JB6 P^+ cells stably expressing dominant-negative p85 subunit of PI3K (JB6^{DNp85}) were exposed to asphalt fumes (20 μg/ml), and phosphorylation of Akt was determined as described above. The experiments were replicated three to four 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 activation and cell transformation. Our results indicate that PI3K is necessary for asphalt fume-induced AP-1 activation as well as cell transformation; blockage of PI3K by chemical inhibitors or expression of dominant-negative PI3K abolishes asphalt fume-mediated AP-1 activation as well as cell transformation. It is interesting to note that blockage of GSK-3β activity partially inhibits asphalt fume-induced AP-1 activation, suggesting that GSK-3β signaling pathway may also contribute to asphalt fume-induced AP-1 activation even though not a primary one. This observation is supported by recent studies demonstrating that GSK-3β regulates AP-1 activity and is involved in skin tumorigenesis (22, 26). Three members of the MAPK family (JNKs, p38 MAPK, and ERKs) are known to be important mediator of AP-1 activity and play a critical role 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 PtnIns(3) phosphate, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (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 PI3K lipid products PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Resting cells contain substantial levels of PtnIns(3) phosphate, but hardly any PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Upon activation of PI3K, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 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-473 without affecting the expression and phosphorylation of PTEN. PTEN antagonizes the action of PI3K activity by dephosphorylating PtdIns(3,4,5)P_3 (42). Thus, asphalt fume-induced Akt

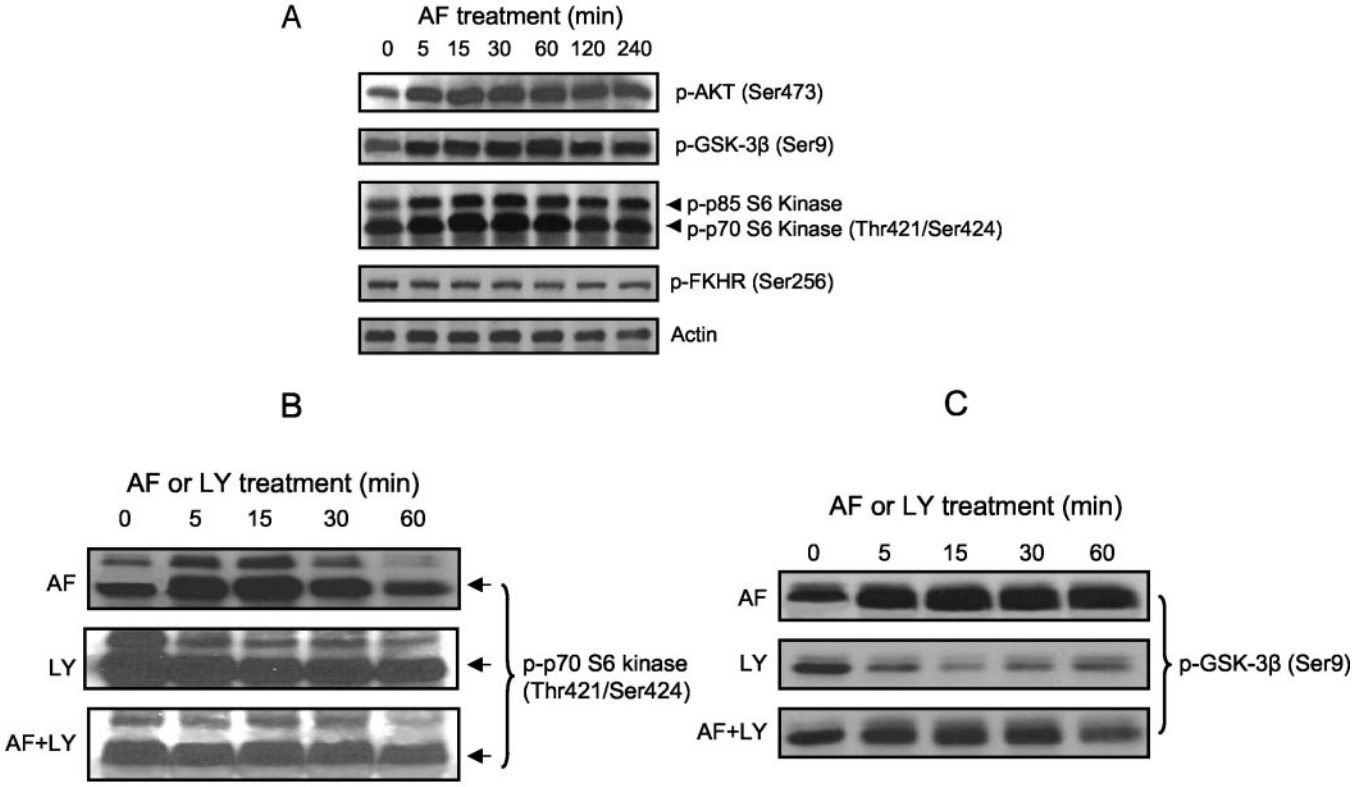

FIG. 6. **Effect of asphalt fumes on Akt-regulated signaling in JB6 P$^+$ cells.** *A*, phosphorylation of downstream signal components of Akt. Phosphorylation of p70/p85 S6 kinase (Thr-421/Ser-424), GSK-3β (Ser-9), and forkhead transcription factor (*FKHR*) (Ser-256) was determined with immunoblots using phosphospecific antibodies. The same blots were striped 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.

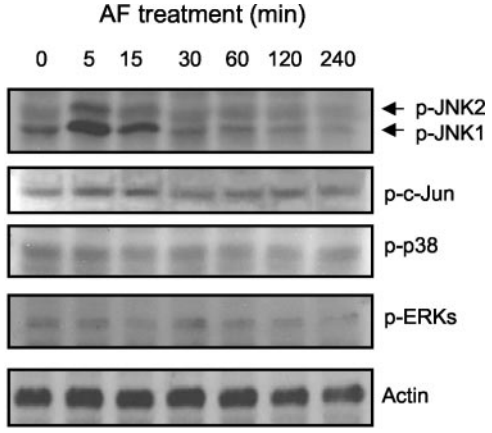

FIG. 7. **Effects of asphalt fumes on the activation MAP kinases in JB6 P$^+$ cells.** Phosphorylation of JNKs, p38 MAPK, and ERKs was investigated with immunoblots using phosphospecific antibodies. The same blots were striped and probed with an anti-actin antibody. The experiments were replicated three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

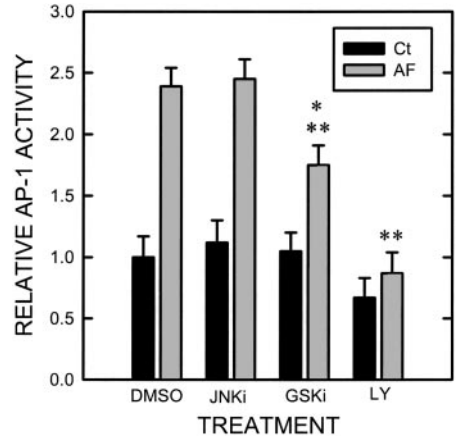

FIG. 8. **Effect of inhibitors for PI3K, JNKs, and GSK-3β on asphalt fume-induced AP-1 activation in JB6 P$^+$ cells.** JB6 P$^+$ cells were pretreated with 10 μM LY294002 (*LY*; PI3K inhibitor), 1 μM D-JNKI (*JNKi*; JNK inhibitor), and 10 μM 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (*GSKi*; 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*, Me_2SO.

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

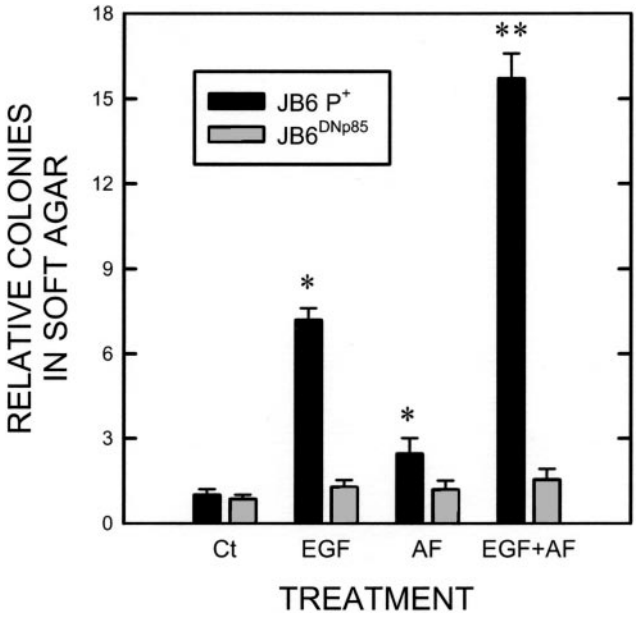

FIG. 9. **Effect of asphalt fumes on anchorage-independent growth of JB6 P$^+$ cells.** JB6 P$^+$ cells or JB6^{DNp85} cells, which were grown in a matrix of soft agar, were exposed to AF (0 or 20 μg/ml) plus EGF (0 or 20 ng/ml). Cell colonies were scored after 14 days of incubation at 37 °C in an atmosphere of 5% CO_2. The number of colonies was expressed as an arbitrary unit relative to the untreated control group. The experiment was replicated four times. *, $p < 0.05$, statistically significant difference from untreated controls. **, $p < 0.05$, statistically significant difference from paired, EGF-treated groups.

asphalt fume-induced phosphorylation of p70S6K/p85S6K.

GSK-3β was named for its ability to phosphorylate, and thereby inactivate, glycogen synthase, a key regulatory process in the synthesis of glycogen. Recent evidence indicates that GSK-3β, a critical figure in many cellular signaling pathways, regulates cell proliferation, survival, and transformation (22). In particular, GSK-3β has been implicated in skin tumorigenesis (26). GSK-3β activity is regulated by serine (inhibitory) and tyrosine (stimulatory) phosphorylation. Asphalt fumes stimulate GSK-3β phosphorylation on Ser-9, and this stimulation is partially inhibited by LY294002, suggesting that the PI3K/Akt pathway only partially contributes to the activation. Serine phosphorylation of GSK-3β may be regulated by multiple molecules. These include Akt, PKC, p90Rsk, and p70S6K (22). Besides Akt, the kinase involved in asphalt fume-stimulated GSK-3β phosphorylation is currently unknown.

In summary, this is the first report demonstrating that exposure to asphalt fumes can activate AP-1 in the skin of mouse and in cultured epidermal cells. Asphalt fumes also promote anchorage-independent growth of mouse epidermal cells, suggesting that exposure to asphalt fumes may favor cell transformation. The action of asphalt fumes is mainly mediated by the PI3K/Akt signaling pathway. PI3K/Akt pathway has been shown to play a critical role in tumor promotion and human malignancy (46, 47). Thus, our study provides evidence showing that asphalt fumes may be involved in carcinogenesis.

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