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Pterostilbene Suppresses Benzo[*a*]pyrene-Induced Airway Remodeling

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ABSTRACT: This study has two novel findings: it is not only the first to demonstrate inflammatory cytokines, which are produced by the bronchial epithelium after exposure to benzo[*a*]pyrene (BaP) and contribute to airway remodeling by increasing human bronchial smooth muscle cells (BSMC) proliferation and migration, but also the first to reveal that pterostilbene, a constituent of grapes and berries, reverses BaP-mediated airway remodeling. Human bronchial epithelial cell lines BEAS-2B and HBE135-E6E7 (HBE) were treated with BaP, and then the condition medium (CM) was harvested, which was then added to BSMC. Cultures of BSMC with BaP-BEAS-2B-CM and -HBE-CM increased BSMC proliferation and migration, which are major features in asthma remodeling. Exposure of BEAS-2B and HBE to BaP caused epithelial cells to produce inflammatory cytokines IL-8, which subsequently induced BSMC proliferation and migration. Moreover, pterostilbene is more potent than resveratrol in suppressing BaP-mediated airway remodeling. This study suggests that pterostilbene is capable of preventing BaP-associated asthma.

KEYWORDS: pterostilbene, resveratrol, benzo[*a*]pyrene, asthma, airway remodeling

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), of which benzo[*a*]pyrene is the most commonly studied and measured, are formed by the incomplete combustion of organic matter. Their presence in the environment is reflected in their presence at detectable levels in many types of uncooked food. In addition, cooking processes can generate PAHs in food. Several studies have been carried out to determine the levels of exposure to PAHs from representative human diets and the proportion of the overall burden of environmental exposure to PAHs that is attributable to the diet.[1−5] Previous studies have pointed to a possible correlation between polycyclic aromatic hydrocarbon exposure and airway diseases, such as asthma.[6−9] However, the effect of polycyclic aromatic hydrocarbon exposure leading to increased airway remodeling is not well understood.

The major features of airway remodeling include loss of epithelial integrity, subepithelial fibrosis, goblet cell and submucosal gland enlargement, increased bronchial smooth muscle mass, and increased angiogenesis.[10] Human bronchial smooth muscle cells (BSMC) play a key role in the modulation of airway tone. In an asthmatic airway, BSMC not only increase secretory and proliferative ability but also migrate to the subepithelial area.[10,11] In addition, BSMC also release proinflammatory cytokines, which are responsible for the progression of asthma pathogenesis. The degree of change in bronchial smooth muscle mass has been directly correlated to asthma severity.[10,11]

Resveratrol and pterostilbene (Figure 1) are naturally occurring compounds belonging to a group called stilbenes, one of the group of phenolics found in grapes, wines, and berries.[12] Recent studies have exhibited that resveratrol and pterostilbene possess antioxidant, antiinflammatory, and anticancer properties.[13−20] In this study, we have not only evaluated the effect of BaP in airway remodeling but also investigated pterostilbene as a potential antidote in the fight against BaP-induced asthma.

MATERIALS AND METHODS

Chemicals. Resveratrol and pterostilbene were purchased from Sigma Chemical (St. Louis, MO), dissolved in dimethyl sulfoxide (DMSO), and stored at −20 °C. Control cultures received the carrier solvent (0.1% DMSO). All other chemicals used were in the purest form available commercially.

Cell Culture and Condition Medium. Two human bronchial epithelial cell lines, BEAS-2B (CRL-9609) and HBE135-E6E7 (HBE, CRL-2741), obtained from ATCC, were used in our study. BEAS-2B was cultured in BEGM medium (Lonza, Walkersville, MD), and HBE135-E6E7 (HBE) was maintained in keratinocyte-serum free medium with 5 ng/mL human recombinant EGF and 0.05 mg/mL bovine pituitary extract (Invitrogen) supplemented with 0.005 mg/mL insulin and 500 ng/mL hydrocortisone. Primary human BSMC were obtained from Lonza and cultured in SmGM-2 smooth muscle medium (Lonza).

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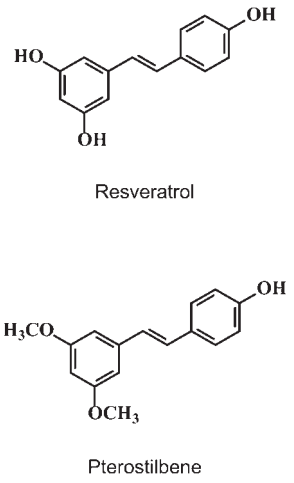

Figure 1. Chemical structures of resveratrol and pterostilbene.

To obtain the various condition media, BEAS-2B and HBE cells (2×10^6/100 mm dish) were treated with various concentrations (0.5, 1, 5, and 10 μM) of BaP for 6 h. After treatment, the medium was replaced and the supernatants were harvested after 24 h of incubation. Depletion of IL-8 from various CMs was performed using anti-IL-8 antibody (2 μg/mL, R&D System) and Sepharose A/G beads following regular immunoprecipitation techniques. Cytokine depletion was confirmed by IL-8 ELISA assay kit.

Enzyme-Linked Immunosorbent Assay (ELISA). Proteins in the culture supernatants were extracted. The levels of IL-8, RANTES, CXCL5, and IL-1β were determined by ELISA-based kits (R&D System Europe, Abingdon, U.K.). ELISAs were performed according to the manufacturer's instructions.

Cell Proliferation. BSMC (4×10^3/well) were plated in 96-well culture plates. After 24 h of incubation, the cells were treated with vehicle control-CM or various condition media for 72 h. BSMC proliferation was determined by premixed WST-1 Cell Proliferation Reagent (Clontech Laboratories Inc., Mountain View, CA) in accord with the manufacturer's instructions. In brief, the cells were incubated with premixed WST-1 Cell Proliferation Reagent for 0.5−4 h. The tetrazolium salt WST-1 was cleaved to a formazan-class dye by mitochondrial succinate−tetrazolium reductase in viable and metabolically active cells, so quantitating the formazan dye by measuring the absorbance at 450 nm in a multiwell plate reader provided measurement of cell proliferation. The percentage of inhibition was calculated using the following formula: % inhibition = $[100 - (OD_t/OD_s) \times 100]$%. OD_t and OD_s indicate the optical density of the test substances and the solvent control, respectively.

Cell Migration Assay. Cell migration assay was carried out using the QCM Chemotaxis 8 μm cell migration assay system (Chemicon, Temecula, CA) (Millipore Corp., Bedford, MA) according to the manufacturer's instructions. Cells were seeded into the migration chamber, and control medium, various condition media, IL-8 depletion condition media were placed in the lower chamber. After cell migration had been allowed for 24 h, cells that had migrated through the membrane were poststained with CyQuant GR dye and examined using a fluorescence plate reader at excitation/emission wavelengths of 485/530 nm.

Microarray and Real-Time RT-PCR. RNA isolation from the cell extracts was performed using the TRIzol reagent (Invitrogen). cDNA was prepared using an oligo (dT) primer and reverse transcriptase (Takara, Shiga, Japan) following standard protocols.

Microarray experiment procedures were carried out following the manufacturer's protocols. Total RNA (1 μg) was amplified by an Agilent Quick Amp Labeling Kit (Agilent Technologies, USA). BaP-BEAS-2B-CM-treated BSMC RNA was labeled by Cy5, and control-CM-treated BSMC RNA was labeled by Cy3 in an in vitro transcription process. Cy-labled cRNA (0.825 μg) was cleaved to an average size of about 50−100 nucleotides by incubation with fragmentation buffer (Agilent Technologies) at 60 °C for 30 min. Equal Cy-labled cRNA was pooled and hybridized to Agilent Whole Human Genome 4x44k oligo microarray (Agilent Technologies, USA) at 65 °C for 17 h. After washing and drying by nitrogen gun blowing, microarrays were scanned by an Agilent microarray scanner (Agilent Technologies, USA) at 535 nm for Cy3 and at 625 nm for Cy5. Scanned images were analyzed by Feature extraction software 10.5 (Agilent Technologies, USA), an image analysis and normalization software used to quantify signal and background intensity for each feature, and the data were substantially normalized by the rank-consistency-filtering LOWESS method.

Real-time PCR was performed using SYBR Green on the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Each PCR reaction mixture contained 200 nM of each primer, 10 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems), and 5 μL of cDNA and RNase-free water in a total volume of 20 μL. The PCR reaction was carried out with a denaturation step at 95 °C for 10 min, then for 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. All PCRs were performed in triplicate and normalized to internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Relative expression was presented using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis. Data were expressed as the mean ± SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($p < 0.05$) between the means of the two test groups were analyzed by Dunnett's test.

RESULTS

Condition Medium of BaP-Treated BEAS-2B and HBE135-E6E7 (HBE) Increased Proliferation and Migration of BSMC. Increase in the mass of BSMC and decreased distance between BSMC and bronchial epithelial cells are important features of the remodeled wall in asthmatic airways.[10] These changes in BSMC can be induced by many factors produced by epithelial cells.[10] We harvested the condition media of BEAS-2B and HBE cells that had been precultured with BaP at different concentrations (0.5, 1, 5, and 10 μM) for 6 h (BaP-BEAS-2B-CM and BaP-HBE-CM) and then assessed the effects of condition media on the proliferation and migration of BSMC. BaP-BEAS-2B-CM and BaP-HBE-CM increased the proliferation of BSMC in a dose-dependent manner after 72 h of incubation. Furthermore, BaP-BEAS-2B-CM and BaP-HBE-CM increased the migration of BSMC in a concentration-dependent manner (Figure 2).

BaP-BEAS-2B-CM and BaP-HBE-CM Increased the Expression of Inflammatory, Adherence, and Chemotactic Factors. Increased inflammatory response of BSMC is a cardinal feature in the development of airway remodeling.[21] We assessed whether BaP increased the inflammatory response through cross-talk of the epithelium and BSMC. As shown in Figure 3A, increased transcription of inflammatory cytokines, including IL-4, IL-6, IL-8, CXCL2, CXCL3, and CXCL5, as determined by microarray of cell extracts, were found in both BaP-BEAS-2B-CM-treated and BaP-HBE-CM-treated BMSC. The data were further confirmed by real-time RT-PCR (Figure 3B). Similarly, BaP-BEAS-2B-CM and BaP-HBE-CM also increased the amount of IL-6 and IL-8 on the protein levels, as determined by ELISA of the supernatants (Figure 3C,D).

BaP Caused an Inflammatory Response in Human Bronchial Epithelial Cells. Increase of inflammatory response in the

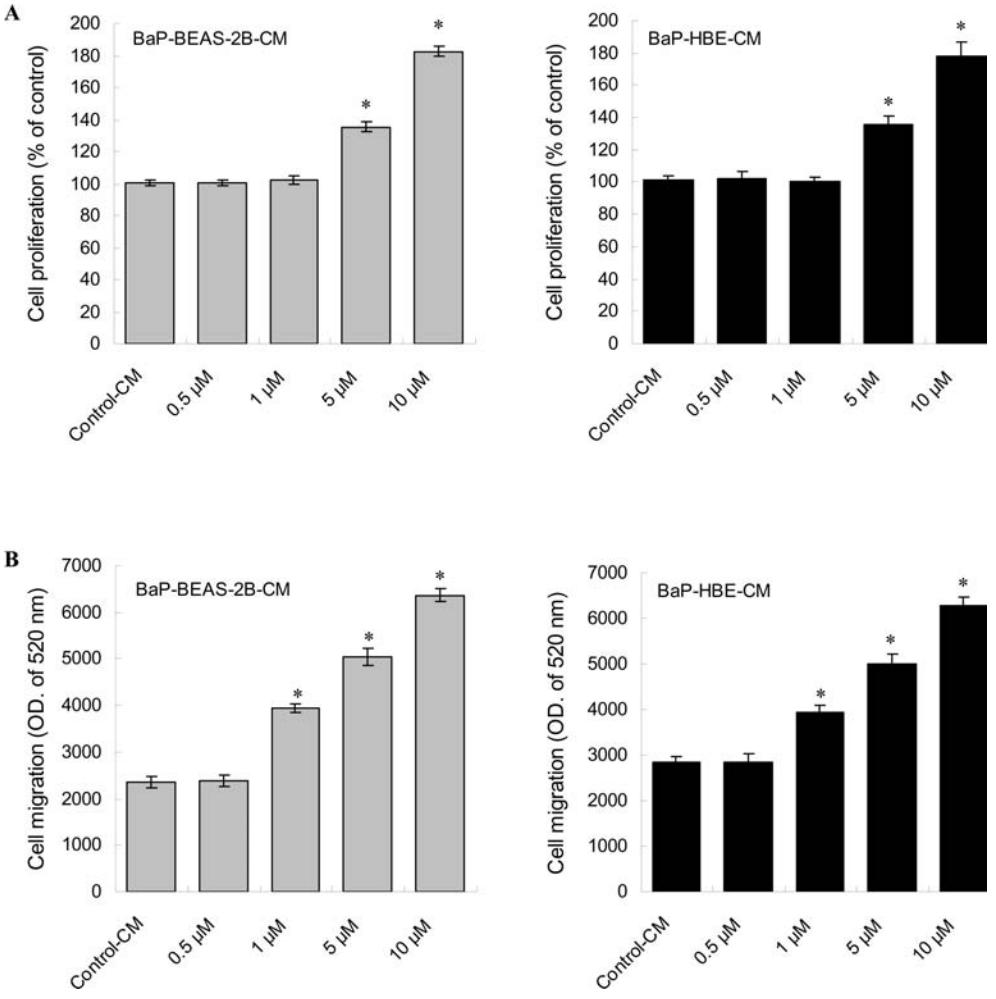

Figure 2. Condition media (CM) from benzo[*a*]pyrene (BaP)-treated BEAS-2B and HBE135-E6E7 (HBE) increased proliferation (A) and migration (B) of bronchial smooth muscle cells (BSMC) in a dose-dependent manner (the condition media produced by epithelial cells treated with higher concentration of BaP showed greater effect in increasing BSMC proliferation and migration). BEAS-2B and HBE cells (2×10^6 cells/100 mm dish) were treated with vehicle control and various concentrations (0.5, 1, 5, and 10 μM) of BaP for 6 h. The medium was replaced with fresh medium, and then cells were harvested after 24 h of incubation. The collected media were defined as BaP-BEAS-2B-CM and BaP-HBE-CM. The effect of BaP-BEAS-2B-CM and BaP-HBE-CM on BSMC proliferation was assessed by WST-1 after 72 h of incubation. BSMC migration was assessed by QCM Chemotaxis cell migration assay system. The *x*-axis indicates the concentration of BaP used to treat BEAS-2B and HBE for producing various condition media. The asterisk indicates a significant difference between control and test groups, $* p < 0.05$.

epithelium plays an important role in the development of asthma.[22] We assessed the effect of BaP on various inflammatory cytokines in human bronchial epithelial cell lines BEAS-2B and HBE135-E6E7 (HBE). As shown in Figure 4A, BaP increased the production of IL-8 in both BEAS-2B and HBE cell lines in a dose-dependent manner after 6 h of treatment. In contrast, BaP failed to affect the secretion of IL-6, eotaxin, and CXCL5 (Figure 4B,C, D, respectively).

IL-8 Is a Major Factor of BaP-BEAS-2B-CM and BaP-HBE-CM in Increasing BSMC Proliferation and Migration. To address the role of IL-8 on BaP-BEAS-2B-CM and BaP-HBE-CM-mediated BSMC proliferation and migration, we depleted IL-8 from BaP-BEAS-2B-CM and BaP-HBE-CM. The successful depletion of IL-8 from BaP-BEAS-2B-CM and BaP-HBE-CM was confirmed by IL-8 ELISA kits (data not shown). As shown in Figure 5A, IL-8 depletion completely reversed the increased proliferation of BSMC induced by BaP-BEAS-2B-CM and BaP-HBE-CM. Similarly, the increase of BSMC migration induced by BaP-BEAS-2B-CM and BaP-HBE-CM was totally abrogated upon depletion of IL-8 (Figure 5B).

Pterostilbene Is More Effective than Resveratrol in Suppressing BaP-Response IL-8 in Mediating Airway Remodeling. The physiological and pathological contribution of BSMC is critical to the process of airway tissue remodeling.[23−25] BSMC proliferation (hyperplasia), increase in BSMC size (hypertrophy), and migration of BSMC toward the epithelium were clearly demonstrated in instances of fatal asthma attacks.[25,26] Because BaP-induced BSMC proliferation and migration have been demonstrated, searching for a potential antidote in the fight against polycyclic aromatic hydrocarbon-induced asthma has become a matter of great urgency. We therefore assessed the effects of resveratrol and pterostilbene, dietary stilbenes from grapes, wines, and berries, on BaP-induced airway remodeling. As shown in Figure 6A, epithelial cell condition media (BaP-BEAS-2B-CM and BaP-HBE-CM) increased the proliferation of BSMC after 72 h of treatment,

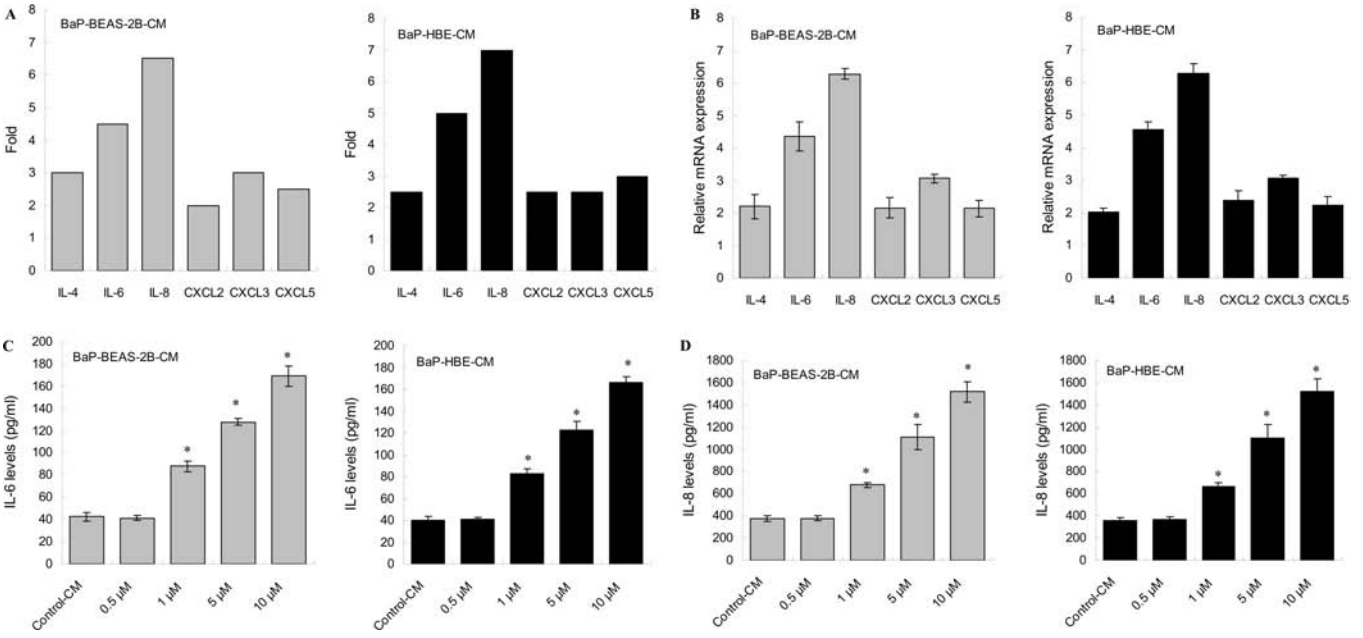

Figure 3. BaP-BEAS-2B-CM and BaP-HBE-CM increased the expression of various inflammatory and chemotactic factors in BSMC on the mRNA level, as assessed by microarray (A) and real-time RT-PCR (B) of cell extracts, and also increased the amount of IL-6 and IL-8 on the protein level, as assessed by ELISA of culture supernatants (C, D). BSMC were treated with BaP-BEAS-2B-CM or BaP-HBE-CM (produced with 10 μM BaP) for 6 h, and mRNA expression was assessed by microarray and real-time RT-PCR of the cell extracts (A, B). BSMC were treated with BaP-BEAS-2B and BaP-HBE-CM for 6 h, and the amounts of IL-6 and IL-8 in the culture supernatants were assessed bys ELISA kit (C, D). The *x*-axis indicates the concentration of BaP used to treat BEAS-2B and HBE for producing various condition media. The asterisk indicates a significant difference between control and test groups, $* p < 0.05$.

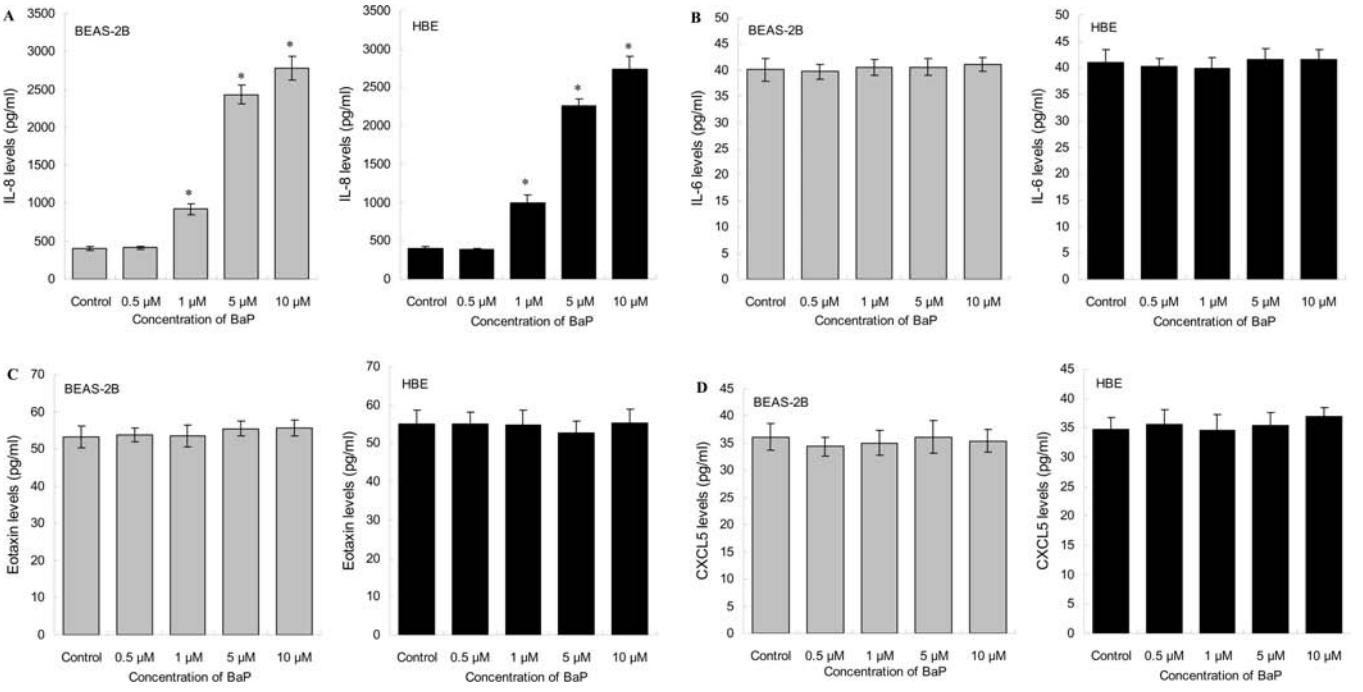

Figure 4. BaP increased production of IL-8 (A) in BEAS-2B and HBE cell lines, whereas it did not affect the levels of IL-6 (B), eotaxin (C), and CXCL5 (D). BEAS-2B and HBE cells were treated with various concentrations of BaP for 6 h. The supernatants were collected and the levels of various cytokines were assessed by ELISA kit. The asterisk indicates a significant difference between control and test groups, $* p < 0.05$.

whereas pretreatment with resveratrol and pterostilbene decreased this effect. Of the two compounds, pterostilbene completely reversed cell growth of BSMC present upon exposure to BaP-BEAS-2B-CM and BaP-HBE-CM. Similarly, increase of BSMC migration triggered by BaP-BEAS-2B-CM and BaP-HBE-CM was totally abrogated upon pterostilbene treatment,

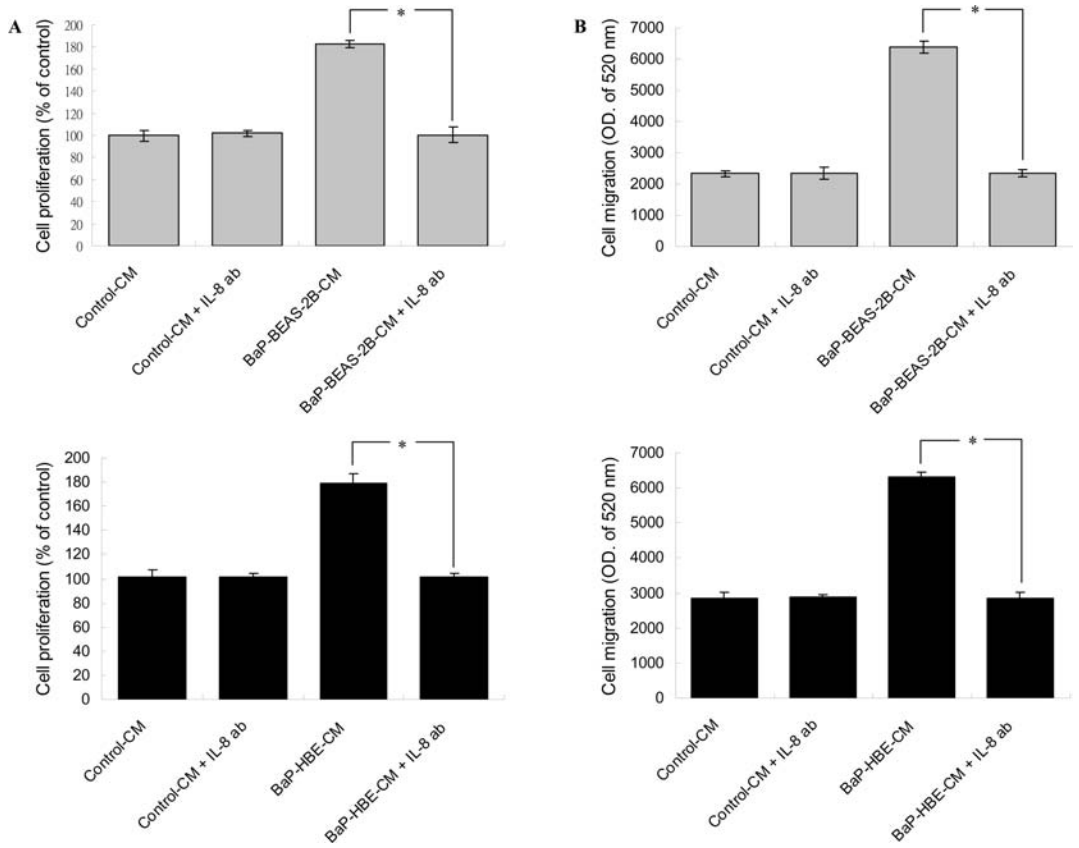

Figure 5. IL-8 is a major factor of BaP-BEAS-2B-CM and BaP-HBE-CM in increasing BSMC proliferation (A) and migration (B). BaP-BEAS-2B-CM and BaP-HBE-CM were produced with 10 μM BaP. IL-8 depletion (indicated as "+IL-8 ab") from the condition media was performed using anti-IL-8 antibody (2 μg/mL) and Sepharose A/G beads, following regular immunoprecipitation techniques. The effect of various condition media on BSMC proliferation was assessed by WST-1 after 72 h of incubation. The effect of various condition media on BSMC migration was assessed by QCM Chemotaxis cell migration assay system. All experiments were performed independently at least three times. The asterisk indicates a significant difference between two test groups, $* p < 0.05$.

whereas treatment with resveratrol alone only partially reversed BSMC migration (Figure 6B).

DISCUSSION

BaP, a member of the polycyclic aromatic hydrocarbon family, is the first detected carcinogen in cigarette smoke.[27,28] It is also found in foodstuffs, industrial waste, and air pollutants. As a major constituent of smoke, BaP is metabolically activated by enzymes in the cytochrome p450 system and becomes capable of binding DNA, forming a DNA adduct, which plays an important role in carcinogenesis.[27−29] Besides carcinogenesis, recent studies also show a variety of effects of BaP, such as inducing apoptosis of bronchial epithelial cells.[30] It may also induce mucin production in airway epithelial cells via a mechanism involving the generation of reactive oxygen species through activation of an aryl-hydrocarbon receptor.[31]

The present study is the first to investigate the interaction of airway epithelium and smooth muscle cells after exposure to PAH. BaP causes human bronchial epithelial cell lines BEAS-2B and HBE135-E6E7 (HBE) to secrete IL-8, which enhances the proliferation and migration of human BSMC. This study is also the first to investigate the effect of pterostilbene in reversing polycyclic aromatic hydrocarbon-induced airway remodeling.

Asthmatic airway remodeling is the pathophysiological modification of the normal airway wall structure, including changes in the composition and organization of its cellular and molecular constituents. These modifications are the major cause of the symptoms associated with decreased pulmonary function.[25] BSMC proliferation (hyperplasia), increase in BSMC size (hypertrophy), migration of BSMC toward the epithelium, and decreased rates of BSMC apoptosis all contribute to the increase in airway smooth muscle mass, a hallmark feature of tissue remodeling. This increase in BSMC mass has been reported to correlate with the severity of asthma.[25,26,32−34] Various mitogens for BSMC proliferation have been identified, including polypeptide growth factors (such as platelet-derived growth factor, insulin-like growth factors, and epidermal growth factor), contractile agonists (such as endothelin-1, substance P, and leukotriene D4), and pro-inflammatory cytokines (such as IL-1β, IL-6, and TNF-α).[35] The bronchial epithelium is known to play a critical regulatory role in the maintenance of airway function and integrity[36,37] and can produce various factors and cytokines/chemokines to regulate other systems or tissues, including BSMC.[10,38] Previous studies have shown that the airway epithelial cells modulate BSMC proliferation via a matrix-metalloproteinase-9-dependent mechanism and a mechanism involving secretion of soluble mediators including IL-6, IL-8, and monocyte chemotatic protein-1.[39] Our results show that BaP caused epithelial cells to produce inflammatory cytokines IL-8, which subsequently induced BSMC proliferation and migration.

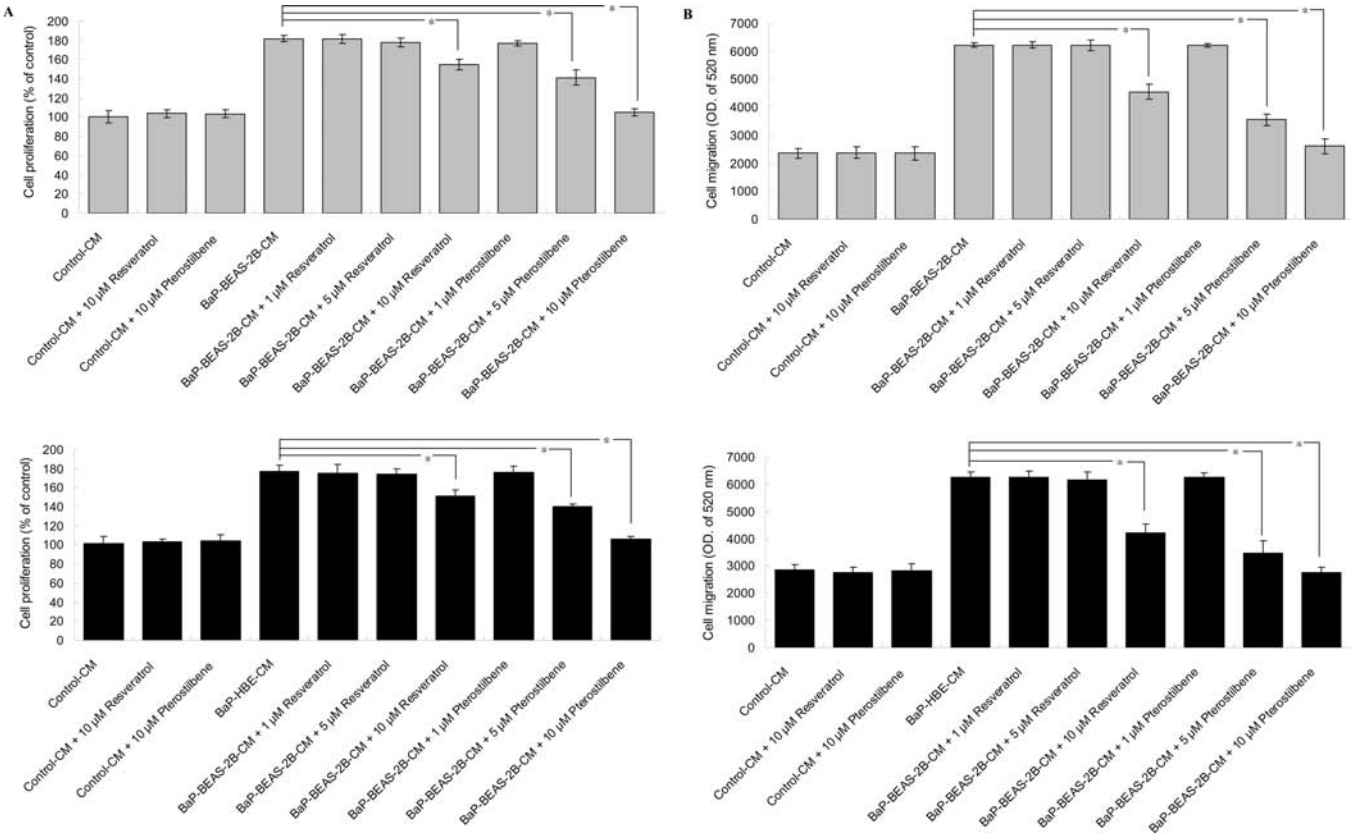

Figure 6. Pterostilbene is more potent than resveratrol in preventing BSMC proliferation (A) and migration (B) induced by BaP-BEAS-2B-CM or BaP-HBE-CM. (A) BSMC were pretreated with various concentrations (1, 5, 10 μM) of resveratrol or pterostilbene for 1 h. Then, control-CM, BaP-BEAS-2B-CM (produced with 10 μM of BaP), or BaP-HBE-CM (produced with 10 μM of BaP), with or without resveratrol or pterostilbene, was added. After incubation for 72 h, cell proliferation was assessed by WST-1 assay. (B) BSMC migration was assessed by QCM Chemotaxis cell migration assay system. BSMC cells were seeded into the top chambers and treated with various concentrations (1, 5, 10 μM) of resveratrol or pterostilbene. Control-CM, BaP-BEAS-2B-CM (produced with 10 μM of BaP), or BaP-HBE-CM (produced with 10 μM BaP) was added to the bottom wells as chemoattractant. After incubation for 24 h, migratory cells were poststained with CyQuant GR dye and examined using a fluorescence plate reader at excitation/emission wavelengths of 485/530 nm. The asterisk indicates a significant difference between two test groups, $* p < 0.05$.

Depleting IL-8 completely reversed BaP-BEAS-2B-CM- and BaP-HBE-CM-induced BSMC proliferation and migration.

Airway inflammation is a major factor in the pathogenesis of asthma,[10,11] and corticosteroids are currently the most effective anti-inflammatory therapy of persistent asthma. Their efficacy in controlling airway inflammation in asthma has been well documented;[25,40] however, their effect on airway remodeling is still debatable. Although some studies have suggested a modest effect of corticosteroids on airway remodeling, the majority of the literature suggests that corticosteroids have little or no effect on tissue remodeling.[25,40−42]

The major effects of resveratrol and pterostilbene, dietary stilbenes from grapes, wines, and berries, include antioxidant, anti-inflammatory, and anticancer properties. Althouth few studies shows anti-inflammatory properties of resveratrol in airway epithelial cells and BSMC, still little is known about its role in the airway; data on the effect of pterostilbene are lacking.[43,44] Our results show that resveratrol and pterostilbene could reverse BaP-induced BSMC proliferation and migration. Therefore, we propose that resveratrol and pterostilbene suppress BaP-response IL-8 in mediating airway remodeling, and further study may be needed to directly identify the mechanism in detail.

In this study, we found that pterostilbene, a natural dimethylated analogue of resveratrol, is more potent than resveratrol in suppressing BaP-induced airway remodeling. This finding is similar to those of other studies with regard to the anticancer effects of pterostilbene.[45] Resveratrol has a low bioavailability to cells, so structural modifications of the resveratrol are needed to increase its bioavailability while preserving its beneficial activities.[15,46,47] Structurally, pterostilbene has a better metabolic stability than resveratrol because it has only one hydroxyl group, whereas resveratrol has three. The dimethyl ether structure of pterostilbene was suggested to enhance its lipophilicity and increase membrane permeability, resulting in better pharmacokinetic profiles than those of resveratrol.[15,48] Nevertheless, the low serum concentration of the active forms due to low bioavailability and rapid and extensive metabolism, according to the pharmacokinetic data mainly from studies about resveratrol, may limit the use of resveratrol or pterostilbene clinically.[48−50] Further study may be needed to define the pharmacokinetic data of pterostilbene in human beings and to define its in vivo effect. Furthermore, as for many drugs used in the treatment of asthma, inhalation may also be considered as another route for delivery of these agents.

Our findings indicate that condition media from polycyclic aromatic hydrocarbon-treated airway epithelial cells stimulate the proliferation and migration of BSMC. It is IL-8 in the condition media that plays a key role of this effect. Because

anti-inflammatory medications have limited impact on airway remodeling,[25,40−42] determining the mechanisms involved in remodeling, including the role of structural cells in asthma, might open new horizons for more effective therapeutic interventions.[25] Our study is the first investigation to provide evidence that pterostilbene has great potential for preventing BaP-associated asthma.

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