Diesel Exhaust Particulates Affect Cell Signaling, Mucin Profiles, and Apoptosis in Trachea Explants of Balb/C Mice

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ABSTRACT: Particulate matter from diesel exhaust (DEP) has toxic properties and can activate intracellular signaling pathways and induce metabolic changes. This study was conducted to evaluate the activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) and to analyze the mucin profile (acid (AB^+), neutral (PAS^+), or mixed (AB/PAS^+) mucus) and vacuolization (V) of tracheal explants after treatment with 50 or 100 μg/mL DEP for 30 or 60 min. Western blot analyses showed small increases in ERK1/2 and JNK phosphorylation after 30 min of 100 μg/mL DEP treatment compared with the control. An increase in JNK phosphorylation was observed after 60 min of treatment with 50 μg/mL DEP compared with the control. We did not observe any change in the level of ERK1/2 phosphorylation after treatment with 50 μg/mL DEP. Other groups of tracheas were subjected to histological sectioning and stained with periodic acid-Schiff (PAS) reagent and Alcian Blue (AB). The stained tissue sections were then subjected to morphometric analysis. The results obtained were compared using ANOVA. Treatment with 50 μg/mL DEP for 30 min or 60 min showed a significant increase ($p < 0.001$) in the amount of acid mucus, a reduction in neutral mucus, a significant reduction in mixed mucus, and greater vacuolization. Our results suggest that compounds found in DEPs are able to activate acid mucus production and enhance vacuolization and cell signaling pathways, which can lead to airway diseases. © 2014 Wiley Periodicals, Inc. Environ Toxicol 30: 1297–1308, 2015.

Keywords: histopathology; DEP; biomarkers; signal transduction; explants; MAPK

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INTRODUCTION

Diesel exhaust particles (DEP) are a major contributor to air particulate mass (PM) in urban areas (Cao et al., 2007). DEP nanoparticles (diameter <100 nm) penetrate deep into the respiratory tract. These particles consist of toxic compounds such as hydrocarbons, sulfur and metals that are adsorbed on the surface of the DEPs (Vermylen et al., 2005; Brook et al., 2010). Air pollution is known to induce a series of cellular responses in the airway epithelium, including mucus hypersecretion and apoptosis (Franco et al., 2009).

Polycyclic aromatic hydrocarbons (PAHs) and nitroarenes are distributed between the particle and gas phase of diesel exhaust. Nitroarenes result from the incomplete combustion of fuel from gasoline burners and diesel engines and have mutagenic and carcinogenic properties (Rosenkranz, 1996; Zwirner-Baier and Neumann, 1999; National Toxicology Program, U.S, 2011). Organic compounds such as PAHs and nitroarenes present in DEPs can lead to the activation of cellular signaling pathways. For example, they can induce the phosphorylation of mitogen-activated protein kinase (MAPK), which initiates an inflammatory response even at nontoxic concentrations (Thomas et al., 1997; Bayram et al., 1998; Ohtoshi et al., 1998; Steerenberg et al., 1998; Boland et al., 1999; Bonvallot et al., 2000). Similarly, *in vitro* and *in vivo* studies have shown that transition metals such as Fe, Cu, Zn, Ni, or V present in DEPs can produce reactive oxygen species (ROS) or catalyze the formation of H_2O_2 by $OH^{\cdot}$ radicals via the Fenton reaction and the Haber-Weiss reaction. These compounds trigger a cellular response mediated by the activation of intracellular signaling pathways (Dreher et al., 1996; Ghio et al., 2002), which culminate in proliferation, cell transformation or cell death (Fantl et al., 1993; Hill and Treisman, 1995).

The extracellular signal-regulated kinase (ERK/MAPK) pathway typically transduces growth factor signals that lead to cell differentiation or proliferation (Marais and Marshall, 1996) and plays an important role in acute lung injury (Schuh and Pahl, 2009), airway remodeling (Guan et al., 2007; Raidl et al. 2007), mucus hyperproduction in chronic airway diseases (Imamura et al., 2004), and allergic airway inflammation (Duan et al., 2004). Moreover, when activated via the classical Ras-Raf-MEK1/2-ERK1/2 pathway, ERK has been implicated in the disruption of the actin cytoskeleton (Barros and Marshall, 2005). Alternatively, the activation of the c-Jun N-terminal kinase (JNK/MAPK) and p38/MAPK pathways results in stress responses, arrested growth, and apoptosis (Xia et al., 1995).

Airways are the first mechanical barrier against air pollution. They are formed by ciliated and mucus-producing epithelial cells (Toledo et al., 2011), and the mucociliary apparatus is the primary defense of the pulmonary system against noxious inhaled agents. Explants of the trachea and lung contain several different cell types, including ciliated, pseudostratified, and columnar epithelial cells; microvilli-covered cells; goblet cells; and undifferentiated and differentiating epithelial cells (Pittet et al., 2010). Therefore, these explants are good models to study the effects of air pollution in respiratory cells.

We hypothesized that exposure to DEPs leads to increased mucus production and apoptosis, which is mediated via the activation of MAPKs. To this end, we evaluated the effects of acute DEP exposure on the phosphorylation of ERK and JNK proteins, mucus production and apoptosis using tracheal explants.

MATERIALS AND METHODS

Composition of Diesel Exhaust Particles (DEP)

In this study, diesel particles were collected in 2005 during one day of routine operation of a bus from the São Paulo metropolitan fleet, which was equipped with a Mercedes Benz MB1620 210-hp engine with a Euro III emission profile. However, the bus did not have electronic fuel injection control, and no post-treatment of the emissions occurred at the exhaust pipe. The diesel particles were stored for toxicological studies at 4°C. The diesel fuel used in Sao Paulo vehicles during this collection period contained 500 ppm of sulfur.

The diesel particle composition was previously characterized by Laks et al. (2008) and Zin et al. (2012). The frequency distribution of particle diameters showed that the diameter of 90% of the DEPs used in this study was <22.60 μm, 50% of the particles were smaller than 6.80 μm, and 10% were smaller than 1.54 μm. The average volume and surface sizes of the particles were 10.02 and 3.60 μm, respectively. For elemental analysis, RX fluorescence spectrometry was used. The concentration of PAHs was evaluated using high-performance liquid chromatography (Laks et al., 2008). The concentrations of the following PAHs were determined: benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenz[ab]anthracene, and indeno [123-cd]anthracene. Table I shows the metal content (ppb) and mean ± standard deviation of polycyclic aromatic hydrocarbons (ng/g) of the intact DEPs (Table I).

Scanning Electron Microscopy

We performed qualitative analyses of the DEP samples diluted in culture medium (50 and 100 μg/mL) using a Field Emission Gun Scanning Electron Microscope, model JEOL JSM-7401F (Tokyo, Japan) at the Institute of Chemistry, University of São Paulo (IQ-USP). The samples were analyzed under magnifications of 10,000 and 40,000×. The samples were filtered using Millipore® filters (0.49 μm pore) and were subsequently dried for 4 h at 37°C. The material was then removed from the filter using double-

TABLE I. Mean ± standard deviation of the polycyclic aromatic hydrocarbons (ng/g) and metal content (ppb) of intact DEP (Laks et al., 2008)

Metals	(Mean ± SD)	PAHs	(ng/g)
Nickel (Ni)	181 ± 37	Naphthalene	49.23
Sulfur (S)	626 ± 416	Acenaphthylene	179.48
Iron (Fe)	74,556 ± 2,2	Fluorene	683.94
Vanadium (V)	37 ± 13	Anthracene	94.73
Lead (Pb)	50 ± 47	Pyrene	12,838.27
Cadmium (Cd)	29 ± 8	Benz[a]anthracene	1,162.73
Chromium (Cr)	161 ± 116	Benzo[b]fluoranthene	789.93
Copper (Cu)	17 ± 1	Benzo[k]fluoranthene	562.28
		Benzo[a]pyrene	1,642.28

sided tape and coated with gold (3 nm coat) to prevent sample degradation.

Determination of the DEP Concentration Used in Tracheal Explant Treatments

We used the MTT assay [3-(4,5 dimethylthiazol-2yl)-2,5 diphenyltetrazolium bromide] (Sigma Chemical, St. Louis, MO) to determine DEP toxicity (Fig. 2). The assay was conducted using BEAS-2B cells (kindly provided by Dr. M. Si-Tahar).

Briefly, the cells were seeded in 96-well plates containing 4×10^4 BEAS-2B cells/well in 180 μL of medium and were cultured for 12 h to allow attachment. DEP suspensions were added at concentrations of 5 μg/mL to 250 μg/mL, and the cells were exposed to the DEPs for 60 min. Next, 5 mg/mL MTT and 90 μL of LHC-9 medium was added, and the cells were incubated for 4 h in a CO_2 incubator. After 4 h, the plates were washed with 100 μL of dimethyl sulfoxide (DMSO) per well and homogenized for 30 min. The ability of the cells to reduce MTT was shown by the production of the formazan, which is an indicator of mitochondrial integrity and cell viability. Then, the plates were read using an ELISA (Enzyme-Linked Immunosorbent Assay) reader (Spectra Max 250, Molecular Devices CA, USA) at 540 nm (Carvalho-Souza et al., 2011). The cell viability values are expressed as the percentage of the absorbance observed for the control cells. This protocol was performed to determine the best concentration of DEPs to use in the tracheal explants.

The exposure concentration was determined based on the surface area of the culture dish. The dish had a diameter of 3.5 cm, corresponding to an area of 9.61 cm^2. We used 150 and 300 μg of particles in 3 mL of media to achieve 50 and 100 μg/mL, respectively. Thus, the exposure concentrations per cm^2 were 15.7 $\mu g/cm^2$ and 31.2 $\mu g/cm^2$, respectively.

Animals

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guiding Principles in the Care and Use of Animals" approved by the Council of the American Physiological Society. Our Institutional Animal Care and Use Committee approved all of the protocols in this study.

Organ Culture

A total of twenty tracheas from 12-week-old adult male BALB/c mice were each divided into four 1-mm pieces. The explants were maintained on plates in Dulbecco's Modified Eagles Medium (DMEM) and Ham's F12 medium (Sigma Chemical) plus supplements and antibiotics (Lankford et al., 2005) for 24 h at 37°C and 5% CO_2. Subsequently, they were divided into five groups containing sixteen samples per group and were treated with 50 and 100 μg/mL of particulate matter from diesel exhaust diluted in DMEM-Ham's F12 for 30 min or 60 min (Sigma Chemical). The control group was maintained in DMEM-Ham's F12 medium (Sigma Chemical) only.

Western Blotting

After treatment, the protein from forty tracheal explants ($n = 8$ in each group) was extracted using lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris pH 7.5, 1 mM Na_3VO_4, 1 mM PMSF, and 2 μg/mL aprotinin) (Sigma Chemical). The tracheal explants were homogenized (Polytron), and the protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). The lysate (40 μg of total protein per lane) was subjected to electrophoretic separation using 10% SDS PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore).

The blots were blocked with 5% BSA in TBST (25 mM Tris pH 7.8, 125 mM NaCl, and 0.1% Tween-20) (Sigma Chemical) for 1 h. The blots were then incubated overnight with specific primary polyclonal antibodies against phospho-JNK1/2 (Calbiochem, San Diego, CA) and phospho-ERK1/2 (Calbiochem). The membranes were washed with TBST and were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma Chemical). The proteins were subsequently visualized in an ImageQuant LAS 4000 (GE Healthcare, Fairfield, CT) using ECL detection reagents (GE Healthcare). To ensure equal protein loading, the same blot was subsequently incubated with anti-MAP Kinase ERK1/2 (Calbiochem) and β-actin (Sigma Chemical). The blots were developed using horseradish peroxidase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated anti-mouse IgG, respectively. After development, the bands were quantified by densitometry.

The amount of protein extracted in each experiment was not enough to perform duplicates or triplicates. Therefore, we were not able to perform statistics**.** The data are densitometric values normalized to β-actin from Figure 3.

Morphometry

The tissues from forty tracheal explants were embedded in paraffin and processed according to routine histological procedures. The samples were analyzed by two investigators who were unaware of the origin of the material. The sections were stained with a combination of periodic acid–Schiff's reagent and Alcian blue (PAS/AB) at a pH of 2.5. With this technique, the neutral and acidic glycoproteins are stained red and blue, respectively (Jones and Reid, 1978). The mucus content (acid, neutral, and mixed) and vacuolization of the respiratory epithelium of the trachea were quantified by conventional morphometry. Using a microscope coupled to a video camera and an image analysis system, we digitized the microscopic image and displayed it on a monitor using a high-resolution video coupled to an eyepiece with a known area containing 884 squares and 3536 points. The volume proportion of the neutral and acid mucus contained in the trachea was determined by point counting (Weibel, 1990; Pires-Neto et al., 2006). Briefly, the number of points hitting on each type of mucosal and nonsecretory area of the epithelium was counted in each field. We then calculated the number of points corresponding to the total area of the epithelium tissue in each field. The same procedure was used to quantify TUNEL-positive cells. Using a Leica DMR microscope attached to both a JVC TK-C 1380 color video camera and an image analysis software system (Image pro-plus), we digitized the microscopic image in a high-resolution video coupled to an eyepiece with a known area. The average thickness of the epithelium was determined by measuring the space between the basal membrane limit and the apical membrane limit (magnification of 1380×).

The quantification of mucus acid (AB^+), neutral mucus (PAS^+), mixed mucus (AB/PAS^+), and the level of vacuolization (V) was determined using the value of the total number of points, N, and the thickness of the epithelium, d, in μm^2 using the following formula:

$$AB^+ \text{ or } PAS^+ \text{ or } AB/PAS^+ \text{ or } V/3.81\ N/d$$

where 3.81 is the area of each point.

TUNEL Technique

The TUNEL technique (Roche, Indianapolis, IN) (Gavriele et al., 1992) was used to determine epithelial apoptosis. After hydration, deparaffinized slides were incubated with proteinase K (20 μg/mL) for 15 min at room temperature. The endogenous peroxidase activity was blocked using 3% hydrogen peroxide in PBS. We conducted primary antibody incubation in a moist chamber at 4°C overnight. After stabilization at room temperature and washing with PBS, the slides were incubated with the secondary antibodies for 1 h at room temperature. After washing with PBS, the slides were incubated with a streptavidin–peroxidase complex for 30 min. The slides were then treated with DAB (diaminobenzidine) substrate. The number of apoptotic cells was calculated from the value of the total number of points, N, and the thickness of the epithelium, in μm^2, using the following formula:

$$\text{apoptotic cells} = 3.81\,N/d$$

where 3.81 is the area of each point.

Statistical Analysis

The data are expressed as the mean ± standard deviation. To compare differences between the control (DMEM/F12) and DEP groups (30 min and 60 min) regarding acid, neutral, and mixed mucus and vacuolization, pairwise multiple comparison procedures (Holm–Sidak method) were used. The Sigmastat v9.0 program was used for the analyses, and the significance level was set at 5%.

RESULTS

Characteristics of DEPs

Two different populations of particles were observed. One population was composed of spherical particles, measuring between 1 and 2 μm in diameter. The second population consisted of very small particles, which formed large agglomerates (Fig. 1).

Determination of the DEP Concentration Used in Tracheal Explants Based on the MTT Assay

The results of the MTT assay using BEAS-2B cells were used to determine the most effective concentration and length of DEP treatment to be used in the tracheal explant experiments. We determined that treatment with 50 μg/mL and 100 μg/mL DEPs for 60 min enhanced metabolic activity (Fig. 2).

Activation of ERK and JNK after Exposure to DEP

Thirty minutes of exposure to 50 μg/mL DEPs led to an increase in the phosphorylation levels of ERK1/2. Furthermore, the phosphorylation levels of JNK1/2 decreased. After sixty minutes of exposure to 50 μg/mL DEPs, the phosphorylation levels of ERK1/2 decreased; however, JNK1/2 phosphorylation increased.

Thirty minutes of exposure to 100 μg/mL DEPs caused the phosphorylation levels of both ERK1/2 and JNK1/2 to increase. After 60 min of exposure to 100 μg/mL DEPs, the

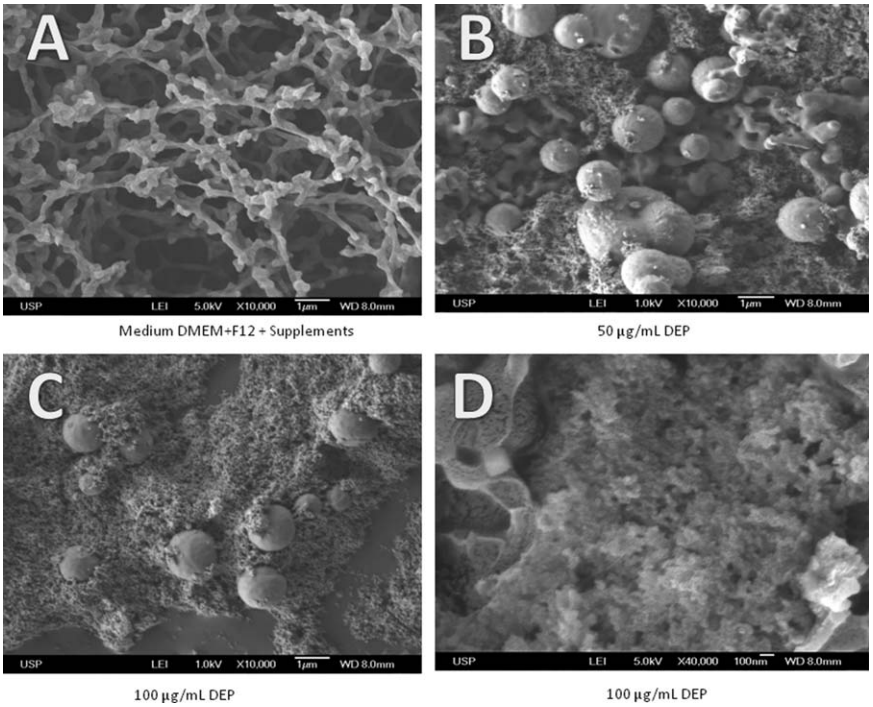

Fig. 1. Scanning Electron Microscopy (SEM) of (A) Dulbecco's Modified Eagles Medium (DMEM) and Hams F12 medium (Sigma Chemical, St. Louis, MO) plus supplements and antibiotics (control) at 10,000× magnification; (B) 50 μg/mL DEPs plus medium at 10,000× magnification; (C) 100 μg/mL DEPs plus medium; and (D) 100 μg/mL DEPs at 40,000× magnification to show smaller sized particles. In this SEM micrograph, it is possible to observe two size populations: one composed of spherical particles measuring between 1 and 2 μm in diameter and a second population of smaller particles that formed aggregates with large agglomerates.

phosphorylation levels of ERK1/2 increased; however, the phosphorylation of JNK1/2 decreased (Fig. 3).

Mucin Profile after DEP Exposure

Figure 4 shows representative photomicrographs of tracheal sections from the five experimental groups stained with AB/PAS. Treatment with 50 μg/mL DEPs for 30 min prompted a significant increase ($p < 0.001$) in the amount of acid mucus. This increase in acid mucus was associated with a decrease in neutral mucus, as shown in Figure 4 ($p < 0.001$). Treatment with 50 μg/mL DEPs caused a significant reduction ($p < 0.001$) in the mixed mucus after 30 min or 60 min. The tracheas treated with 50 μg/mL DEPs for 30 min displayed greater vacuolization ($p < 0.001$), which may be linked to the release of higher amounts of neutral mucus. No statistically significant differences were observed in the explants treated with 100 μg/mL DEPs for 30 min or 60 min compared with the control group.

Apoptosis

Overall, the groups treated with 50 μg/mL DEPs for 30 min or 60 min showed a statistically significant increase in the number of TUNEL-positive cells when compared with the controls ($p < 0.001$). The group treated with 100 μg/mL DEPs for 30 min had a higher number of TUNEL-positive cells ($p < 0.001$) than all of the other groups. The group treated with 100 μg/mL DEPs for 60 min had a lower number of TUNEL-positive cells compared to the other DEP groups; however, considerable epithelial loss was observed in this group (Fig. 5). Eight tracheal explants embedded in

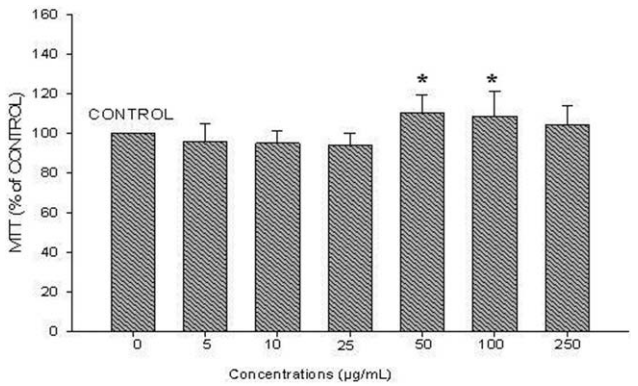

Fig. 2. MTT assay (tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide) of BEAS-2B cells exposed to different concentrations (5 μg/mL to 250 μg/mL) of DEPs for 60 min.

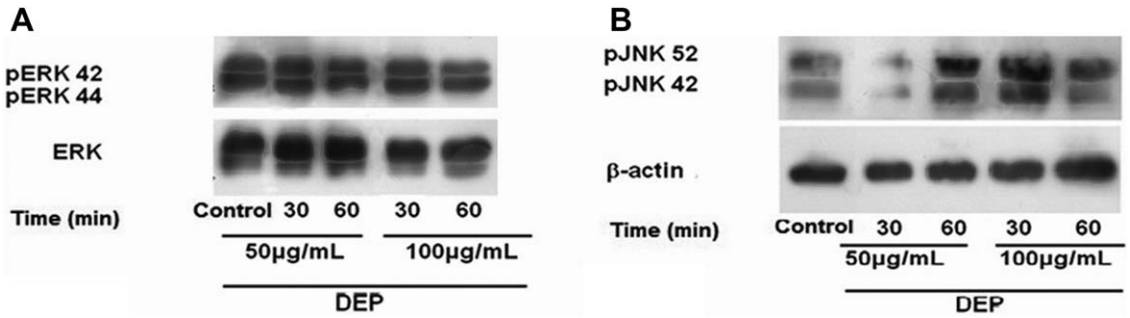

Fig. 3. Effect of DEPs on the phosphorylation of ERK1/2 (A) and JNK (B) in tracheal explants. The tracheal explants were exposed to one of two concentrations of DEPs (50 or 100 μg/mL) for 30 min or 60 min.

each slide were used for each group. The integrity of the epithelium for most of the explants was not adequate to permit the measurement of the TUNEL-positive cells with the desired accuracy. Therefore, we analyzed 3–4 tracheal explants on each slide for all of the groups (Fig. 4).

DISCUSSION

In this study, we observed an increase in the concentration of phosphorylated ERK and JNK in tracheal explants shortly after exposure to DEPs. These findings were associated with alterations in the mucus profile of the tracheal cells, including cellular vacuolization, which suggests mucus extrusion The DEP-exposed cells demonstrated increased apoptosis.

Studies performed using lung and tracheal explants enable the analysis of the response of different cell types to a given stimulus, which differs from the study of cell lines (Pittet et al., 2010). Tracheal explants display many key features of *in vivo* airways, such as mucus coverage, mucociliary clearance and cell structure (Kitson et al., 1999). Moreover, tracheal tissues add a further dimension by providing the opportunity to examine the interactions between the epithelium and the underlying airway mucosal structural cells *ex vivo* (Abeynaike et al., 2010). In addition, tracheal explants have been previously used to investigate the effects of mineral dust and air pollution particles on tracheal function (Dai et al., 1998; Dai et al., 2003). Therefore, tracheal explants may be useful for the study of complex mixtures in numerous substances, such as diesel exhaust. These substances contain various toxic compounds and have physical characteristics that can result in the materials being retained in upper airways (by inflammatory cells) or reaching the lungs.

Previous studies using animals have demonstrated that air pollution modifies the epithelial cell profile by increasing the number of mucus-producing cells and modifying the physical and chemical characteristics of mucus (Saldiva et al., 1992). Ishihara and Kagawa (2003) observed qualitative changes in the types of glycoproteins present in the mucus granules of goblet cells, and these changes depended on the concentration and the length of exposure to particulate air pollution involving diesel exhaust.

In our study, we verified the activation of the MAPK pathway (ERK and JNK) in tracheal explants exposed to particulate matter from diesel exhaust. The MAPKs are involved in metabolic disorders, proliferation and apoptosis. ERK was the first MAPK cascade protein demonstrated to be important in different cellular signaling processes such as growth, proliferation, and survival. There are two predominant ERK isoforms (ERK1 and ERK2), which are referred to as p42/p44 MAP kinases (Boulton et al., 1990, 1991). ERK1 and ERK2 phosphorylate active substrates in all cellular compartments, including membrane proteins (CD120a, Syk, and calnexin), nuclear substrates (SRC-1, Pax6, NF-AT, Elk-1, MEF2, c-Fos, c-Myc, and STAT3), cytoskeletal proteins, and kinases (Chen et al., 2001). The abnormal regulation of MAPK pathways has been reported in a wide range of diseases including many cancers and pulmonary diseases, such as asthma (Duan et al. 2004; Pelaia et al. 2005), emphysema (Mercer et al., 2004), and COPD (Renda et al., 2008).

The ERK1/2 pathway controls the activity or abundance of the BCL-2 protein family, cytochrome *c* from mitochondria, the activation of caspases and cell death (Balmanno and Cook, 2009). According to Hewson et al. (2003), in airways, ERK is a critical tyrosine kinase involved in the induction of MUC5AC expression and goblet cell metaplasia in response to a variety of stimuli. Amara et al. (2007) showed that ERK1/2 mediates the activation of NOX4 and MMP-1 in human lung epithelial cells exposed to DEPs, and Pourazar et al. (2008) showed that ERK activation transduces proliferative and differentiation responses in the bronchial epithelium. Li et al. (1998) indicated that ERK/MAPK activation via the c-src-MAPK-pp90rsk pathway can also be a sufficient signal for NF-κB activation and MUC2 in epithelial cells. Furthermore, Li et al. (2012) showed that a significant increase in phosphorylated ERK induces MUC5AC expression in bronchial epithelial cells.

The c-Jun NH2-terminal kinase (JNK) pathway includes mitogen-activated protein kinases that are activated primarily by cytokines and exposure to many

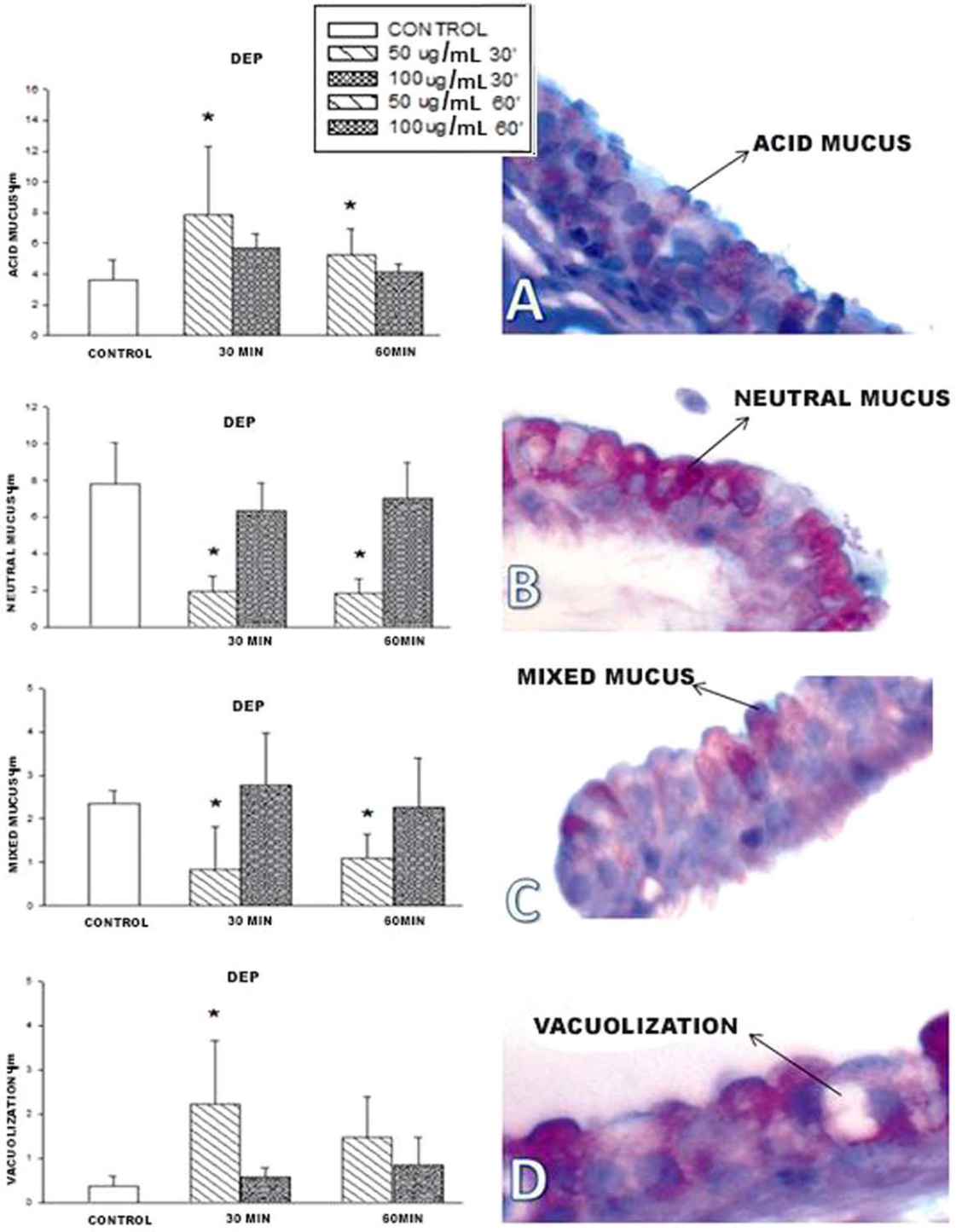

Fig. 4. Mucin-occupying area in the tracheal explants of BALB/c mice exposed to 50 μg/mL or 100 μg/mL DEP. (A) An increase in the content of acid mucus after treatment with 50 μg/ml DEP for 30 min or 60 min (*$p < 0.001$) and (B) a reduction in neutral (*$p < 0.001$), and (C) mixed mucus (*$p < 0.001$) were observed. (D) A significant increase in vacuolization (*$p < 0.001$) was observed only at a concentration of 50 μg/mL after 30 min. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

stresses, including changes in the physical and chemical properties of the environment. Additionally, this pathway contributes to apoptotic signal transduction (Davis, 2000; Weston and Davis 2003). Tournier et al. (2000) implicated JNK in apoptosis because $jnk1^{-/-}jnk2^{-/-}$ mice were resistant to apoptosis induced by UV irradiation,

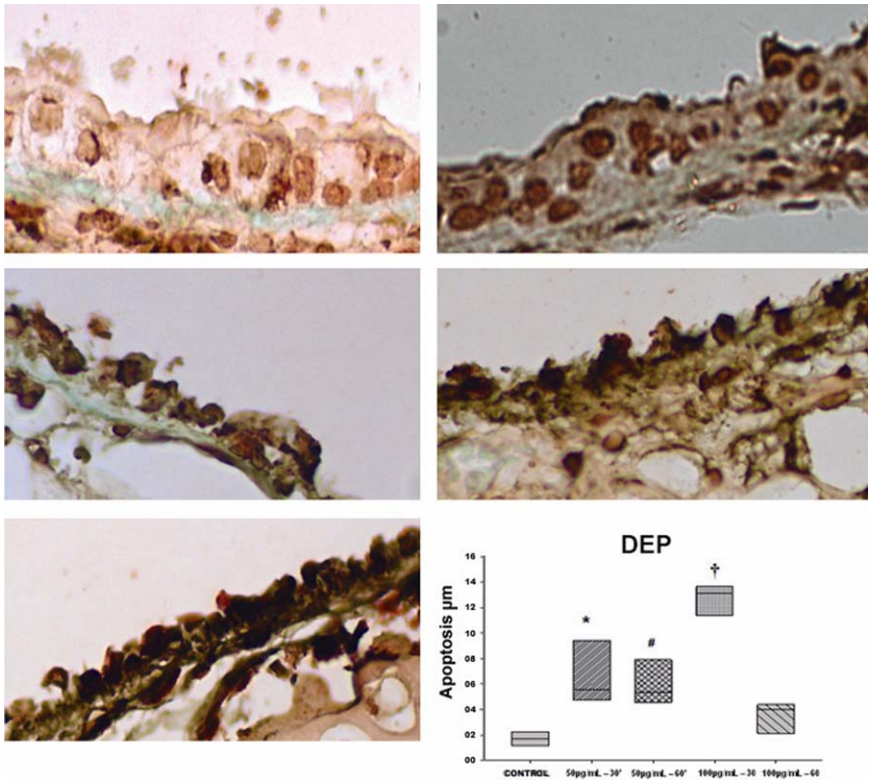

Fig. 5. Images and graphic representation of the proportion of cells undergoing apoptosis after treatment with 50 or 100 μg/mL DEPs for 30 min or 60 min. Statistically significant differences in apoptosis were observed after treatment with 50 μg/mL DEPs for 30 min (*$p = 0.004$), 50 μg/mL DEPs for 60 min (#$p = 0.006$), and 100 μg/mL DEPs for 30 min (†$p < 0.001$ compared with the control group. (B) Light microscopy of cells stained using the TUNEL assay depicts dark brown cells undergoing apoptosis (40×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

anisomycin and MMS. According to Davis (2000) and Weston and Davis (2007), JNK is important in other forms of cell death, including necrosis and autophagy because the mitochondria are a primary target of JNK-mediated pro-apoptotic signaling.

JNK-signaling studies in mammals suggest that JNK induces apoptosis by modulating the pro-apoptotic Bcl-2 family protein BIM (Lei et al., 2003; Nateri et al., 2005). Sakurai et al. (2006) also support a role for JNK in tumor development. Thus, this pro-oncogenic role of JNK may be related to its known ability to promote proliferation: the deregulation of JNK has been implicated in cancer (Davis, 2000; Chang and Karin, 2001; Shaulin and Karin, 2002; Weston and Davis, 2007).

Other properties of JNK activation have been described by Jaeschke et al. (2005). These authors showed that JNK2 controls the Th1/Th2 balance of the immune response, thereby protecting against autoimmune disease. JNK1 is implicated in autoimmune disease, where it plays an important role in regulating the expression of the anti-inflammatory cytokine IL-10. In addition, Choi et al. (2010) observed mucus overproduction and MUC5AC protein expression associated with JNK expression in the epithelia of airways exposed to smoke.

DEPs have been shown to induce apoptosis in airway cells via MAPK activation and oxidative stress (Franco et al. 2009). The activation of JNK has been proposed to have a role in promoting apoptosis. The chemical composition of DEPs is important in this process due to the presence of metals and PAHs. These compounds activate several signaling proteins during biotransformation and produce ROS, which induce the activation of AP-1, MEK-1, ERK, JNK, NF-κB and p53 and cause DNA damage. The activation of several signaling pathways has been shown to be mediated by the inhibition of protein tyrosine phosphatases, and the stimulation of tyrosine residue phosphorylation triggers or potentiates cell apoptosis (Ye et al., 1999; Huang et al., 2000; Au et al., 2006).

According to Reardon et al. (1999) and Shen et al. (2003), the pro-survival ERK and pro-apoptotic JNK pathways act in a dynamic equilibrium, with the ERK pathway acting to inhibit the JNK pathway or vice versa. This interaction could possibly explain the increases and decreases in ERK and JNK in our measurements at 30 min to 50 or 100 μg/mL DEPs.

However, we cannot exclude that other pathways leading to apoptosis could have influenced our results. According to Xia et al. (2004), the aromatic fraction of DEPs can directly

induce mitochondria swelling and depolarization leading to calcium overload in the matrix and causing cellular death. Moreover, the high levels of non-carcinogenic metals (i.e., cobalt, lead, iron, and zinc) present in DEPs have often been shown to provoke the production of ROS (e.g., H_2O_2), which leads to apoptosis through the mitochondrial pathway.

Lemos et al. (1994) and Pires-Neto et al. (2006) showed an increase in the concentration of acid mucus in the nasal epithelium of rats exposed to urban particulate matter. Yoshizaki et al. (2010) showed that exposure of mice to diesel particulate matter for 60 days resulted in an increase in Muc5ac protein expression in the lungs and an increase in acid mucus in the nasal epithelium. Mucin acidification induces alterations in the physico-chemical properties of mucus, with an increase in viscosity that damages mucociliary clearance and the defenses of the respiratory epithelium (Lemos et al., 1994; Pires-Neto et al., 2006; Yoshizaki et al., 2010). In this study, we also showed that an increase in acid mucus occurs primarily after treatment with a low concentration of DEPs (50 μg/mL), which may be due to a ceiling effect. In addition, other factors, such as apoptosis, may have influenced the results (Fig. 2). The *in vivo* secretion of mucins, which is usually initiated by a secretagogue, occurs rapidly, within seconds to minutes; however, the genetic regulation and biosynthesis of mucins requires hours (Neutra et al., 1987; Verdugo, 1991; Davis, 2002). In our study, the exposure was conducted for a maximum of one hour, which may explain the hypersecretion of produced mucin.

In a mouse model of smoke inhalation injury, Choi et al. (2010) showed that mucus overproduction is associated with an increase in epithelial Muc5ac protein expression, which is dependent on the activation of the JNK pathway. These results suggest that the JNK pathway may be a potential target for regulating mucus overproduction. ERK is a critical tyrosine kinase involved in the induction of MUC5ACexpression and goblet cell metaplasia in response to a variety of stimuli (Hewson et al., 2003; Songs et al., 2005). Our results corroborate these previous studies, particularly those using a DEP concentration of 50 μg/mL.

It is possible that the vacuoles observed in our study are the result of endocytosis of the DEPs by tracheal cells, as observed by Geiser (2002) and Bao et al. (2007) in CHO-K1 cells. However, we cannot exclude the possibility that the vacuoles may represent mucus extrusion. Mucin secretion *in vivo* is typically initiated by a secretagogue, and its extrusion can occur within minutes (Rose and Voynow, 2006). In the present study, the explants were maintained for 24 h in culture medium after 30 or 60 min of DEP exposure, and DEPs may have acted as a secretagogue. We showed increased vacuolization at the 30 min timepoint and a slight increase at the 60 min timepoint after treatment with 50 μg/mL DEPs, which can be explained by the observed extrusion of neutral and mixed mucus. ERK activation was present at 30 min and may be responsible for the depolymerization of actin and the release of vesicles by the secretory cells. JNK activation, which inhibits the polymerization of microtubules and induces apoptosis, was observed after treatment for 60 min with 50 and 100 μg/mL DEPs. According to Barros and Marshall (2005) and Tricker et al. (2011), ERK1/2 signaling has been shown to lead to the disruption of actin stress fibers and the loss of focal adhesion by several mechanisms. MEKK1 is a mitogen-activated protein kinase kinase kinase (MAP3K) that activates JNK and is required for microtubule inhibitor-induced apoptosis in B cells. Active cytoskeletal rearrangements have also been shown to accompany many events associated with vesicle transport and fusion in a variety of cell systems (Koffer et al., 1990; Vitale et al., 1995; Lang et al. 2000).

We have used BEAS-2B as a surrogate of tracheal epithelial cells because these cells respond to diesel toxicity. We believe both cell types have similar responses to cytotoxicity. However, to our knowledge, there are no studies comparing the effects of DEPs in BEAS-2B and tracheal explants. Future studies should be performed to clarify these points.

In Sao Paulo, doses of 200 $\mu g/m^3$ PM10 are not uncommon in traffic corridors (Yoshizaki et al., 2010). If we assume that humans breathe 6 L/min, the volume inspired in one hour will be 360 L, equivalent to 0.36 m^3, corresponding to 70 μg of inhaled PM. Considering that particle retention in trachea is approximately 20%, 14 μg is retained in the trachea/hour. Healthy subjects produce a mean of 50 mL of mucus/day 10–100 mL (Rubin, 2002). Thus, the amount of mucus produced in the trachea will be approximately 2.0 mL in 1 h. Thus, the expected concentration of PM in the mucus lining the trachea in one hour is 7 μg/mL/h. In our study, we used DEP concentrations of 50 and 100 μg/mL for 30 min or 1 h. The doses used here would then correspond to 12–14 h of exposure to urban air pollution. Other similar studies have used up to 500 $\mu g/cm^2$ (Dai and Churg, 2000; Dai, et al., 2002; Dai, et al., 2003).

The concentrations used in our study were low doses and were used to show the toxic effects of DEPs in airways and the risks of exposure to these concentrations. We observed that higher concentrations of DEPs in the culture medium tended to form large agglomerates. It is possible that at a concentration of 50 μg/mL, the DEPs would be more dispersed in the medium and penetrate epithelial cells more easily than at the 100 μg/mL concentration. Accordingly, the stress response to injury in the form of acid mucus was more pronounced in the cells exposed to the lower concentration of DEPs.

In conclusion, these results suggest that compounds found in particulate matter from diesel exhaust activate cell signaling pathways, thereby inducing the production of acid mucus, vacuolization and apoptosis in tracheal explants.

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