Eugenol attenuates pulmonary damage induced by diesel exhaust particles

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Eugenol attenuates pulmonary damage induced by diesel exhaust particles. J Appl Physiol 112: 911–917, 2012. First published December 22, 2011; doi:10.1152/japplphysiol.00764.2011.—Environmentally relevant doses of inhaled diesel particles elicit pulmonary inflammation and impair lung mechanics. Eugenol, a methoxyphenol component of clove oil, presents in vitro and in vivo anti-inflammatory and antioxidant properties. Our aim was to examine a possible protective role of eugenol against lung injuries induced by diesel particles. Male BALB/c mice were divided into four groups. Mice protective role of eugenol against lung injuries induced by diesel particles. For this purpose, lung mechanics, architecture, as well as oxidative stress, apoptosis, and inflammatory cell influx were used as markers.

**MATERIALS AND METHODS**

**Particle analysis.** The particles used in this work belonged to the same batch as those whose composition (metals and organic content) was previously reported (13). They were collected as follows: a particle trap device was adapted to the exhaust pipe of a bus from the public transportation fleet of São Paulo, equipped with a Mercedes Benz MB1620, 210-horsepower engine, without electronic control of
fuel injection, running with diesel containing 500 parts/million sulfur. This particular type of bus was chosen because it is the most frequent one operating in São Paulo during particle collection, based on the information given by the municipality. Briefly, a mesh made of stainless steel was inserted into the exhaust pipe line of the bus. Diesel particles were collected after 1 day of routine operation of the bus and stored for toxicological studies.

The distribution of particle sizes, as measured by their volume and surface, and the diameters encompassing 90%, 50%, and 10% of the particulate matter were determined by laser diffraction (Long Bench Mastersizer, Malvern Instruments, Malvern, UK). The particulate matter was visualized by scanning electron microscopy (JEOL 5310, Tokyo, Japan). The analysis was performed at the Laboratory of Technological Characterization, Department of Mining and Petroleum Engineering, Polytechnic School, University of São Paulo, São Paulo, Brazil.

**Animal preparation.** Forty male BALB/c mice (20–25 g) were divided into four groups. In control (CTRL) and diesel (DIE) groups, animals were intranasally (in) instilled with either 10 μl of sterile saline solution (0.9% NaCl, 37°C) or 15 μg of diesel particles (in 10 μl of saline), respectively, and treated 1 h later with sterile saline solution (0.9% NaCl) and Tween 1% byavage (total volume = 0.25 ml). Just before each intranasal administration, the diesel particles were resuspended in saline by a vortex agitator (AP-56, Phoenix , Araraquara, Brazil). In eugenol (EUG) and diesel plus eugenol (DEUG) groups, mice received 10 μl of saline or 15 μg of diesel particles (in 10 μl of saline in), respectively, as described above, and treated 1 h later with saline, 164 mg/kg of eugenol (minimum content: 99%, Vetec Química Fina, Rio de Janeiro, Brazil) and Tween 1% (total volume = 0.25 ml). All animals were analyzed 24 h after gavage. During gavage, mice were anesthetized with sevoflurane. No deaths owing to this procedure resulted.

All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “APS’s Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training.” The experiments were approved by the Ethics Committee on the Use of Animals, Health Sciences Center, Federal University of Rio de Janeiro (protocol IBCCF 046). Twenty-four hours after gavage, the animals were sedated with diazepam (1 mg ip) and anesthetized with pentobarbital sodium (20 mg/kg body wt ip), paralyzed with pancuronium bromide (0.1 mg/kg body wt iv), and mechanically ventilated (Samav VR15, Universidad de la Republica, Montevideo, Uruguay) with a frequency of 100 breaths/min, tidal volume of 0.2 ml, flow of 1 ml/s, and positive end-expiratory pressure (PEEP) of 2 cmH2O. The anterior chest wall was surgically removed.

A pneumotachograph (1.5-mm ID, length = 4.2 cm, distance between side ports = 2.1 cm) was connected to the tracheal cannula for the measurements of airflow (V’), and changes in lung volume (tidal volume) were obtained by flow signal digital integration. The pressure gradient across the pneumotachograph was determined by means of a Validyne MP45-2 differential pressure transducer (Engineering Corp, Northridge, CA). The flow resistance of the equipment (Req), tracheal cannula included, was constant up to flow rates of 26 ml/s and amounted to 0.12 cmH2O·ml⁻¹·s⁻¹. Equipment resistive pressure (Res·V’) was subtracted from pulmonary resistive pressure so that the present results represent intrinsic values. Transpulmonary pressure (Pn) was measured with a Validyne MP-45 differential pressure transducer (Engineering Corp).

Lung resistive (ΔP1) and viscoelastic/inhomogeneous (ΔP2) pressures, total pressure drop after flow interruption (ΔPot = ΔP1 + ΔP2), lung static (Est) and dynamic (Edyn) elastances, as well as ΔE (Edyn-Est) were computed by the end-inflation occlusion method (2, 3). ΔP1 selectively reflects airway resistance in normal animals and humans, and ΔP2 reflects stress relaxation or viscoelastic properties of the lung, together with a small contribution of time-constant inhomogeneties (pendelluft) (3, 31). Lung mechanics were measured 10–15 times in each animal.

**Histology.** Heparin (1,000 IU) was intravenously injected immediately after the determination of respiratory mechanics. The trachea was clamped at end expiration, and the abdominal aorta and vena cava were sectioned, yielding a massive hemorrhage that quickly euthanized the animals. The lungs were perfused with saline and then removed en bloc. The right lung was isolated, frozen in liquid nitrogen, and stored for further analysis; the left lung was fixed in Millonig formaldehyde. After 24 h of fixation, specimens were dehydrated in alcohol and embedded in paraffin. Later on, 4-mm-thick slices were cut and stained with hematoxylin-eosin. For immunohistochemistry or TUNEL assay, the slices were cut and transferred to immunoslides (EasyPath, cod EP-51-30185, Erviegas, São Paulo, Brazil).

Morphometric analysis was performed with an integrating eyepiece with a coherent system with 100 points and 50 lines coupled to a conventional light microscope (Axioplan, Zeiss, Oberkochen, Germany). The point-counting technique was used across 10 random noncoincident microscopic fields to evaluate the fraction area of collapsed alveoli (expressed as collapsed alveoli/number of alveoli) (>200), as well as the amount of polymorphonuclear (PMN) and mononuclear (MN) cells (expressed as cells/pulmonary tissue area) (<×1,000) (41). Two investigators, who were unaware of the origin of the coded material, examined the samples microscopically.

**TUNEL assay.** Apoptotic cells in pulmonary tissue from all groups were evaluated by the terminal deoxytransferase uridine triphosphate nick-end-labeling technique (TUNEL), which detects DNA strand breaks by enzymatic labeling of the free 3’-OH termini with modified nucleotides (ApopTag Peroxidase in situ apoptosis detection kit, Chemicon International, Temecula, CA). The reaction was performed according to the manufacturer’s instructions.

**Immunohistochemistry.** Immunohistochemistry for activated caspase-3 was done using cleaved caspase-3 (Asp-175) antibody (Cell Signaling Technology, Beverly, MA, catalog no. 9661, at 1:100 dilution) as recommended by the manufacturer. Paraffin-embedded sections were dehydrated and hydrated. Immunostaining was performed according to a streptavidin-biotin peroxidase technique using the StreptABComplex-HRP Duet System (Dako Denmark, Glostrup, Denmark). The immunoreactivity was visualized after incubation with freshly prepared 3,3’-diaminobenzidine (Dako Denmark). Slides were counterstained with Harris’ hematoxylin. The endogenous peroxidase of lung tissue was blocked with 3% hydrogen peroxide for 30 min in the dark before exposure to primary antibodies. Negative controls were carried out by omitting the primary antibody. The sections were coverslipped in Entellan (Merck, Darmstadt, Germany) and analyzed by light microscopy.

**Quantification of cell death.** The assessments of the number of apoptotic cells by TUNEL assay and immunohistochemistry (caspase-3 activation) were performed using captured high-quality images (2,048 × 1,536 pixels) obtained with an image analysis system composed of a digital camera (Evolution, Media Cybernetics, Silver Spring, MD) coupled to a light microscope (Eclipse 400, Nikon, Tokyo, Japan) and the software Image Pro Plus 4.5.1 software (Media Cybernetics). In each group, 20 high-power photomicrographs were obtained using ×400 magnification from areas containing the largest amount of cells (hot spot), and the reactive nuclei for both TUNEL and cleaved caspase-3 were manually counted in all captured images. Reactive nuclei were considered as those that were brownish independent of their intensities. Negative cells remained bluish due to the slight hematoxylin counterstain. The ratio between positive and total amount of cells was calculated for each one of the 20 microscopic fields. Median, interquartile (25–75%), and 10–90% ranges were calculated from these 20 ratios for TUNEL and cleaved caspase-3. A single observer performed morphological measurement blindly.

**Catalase activity assay.** To determine catalase (CAT) activity, right lung tissue was sonicated in 50 mM phosphate buffer, and the
resulting suspension was centrifuged at 3,000 g for 10 min. The supernatant was used for enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm. Final activity values were corrected by protein amount in each sample and expressed as units per microgram of protein. Catalase activity was measured as previously described (1).

**Determination of malondialdehyde.** As an index of lipid peroxidation we used the formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction as previously described (7). Briefly, samples from lung homogenates were mixed with 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid. The samples were then heated in a boiling water bath for 30 min. TBARS were determined by absorbance at 535 nm and were expressed as malondialdehyde (MDA) equivalents (MDA nmol/mg protein).

**Protein determination.** The amount of protein in CAT and MDA assays was determined using the Lowry technique (15).

**Statistical analysis.** SigmaPlot11 statistical package (SYSTAT Software, Chicago, IL) was used. When percentage values were to be tested, they first underwent an arcsine transformation. The normality of the data (Kolmogorov-Smirnov test with Lilliefors’ correction) and the homogeneity of variances (Levene median test) were tested. If both conditions were satisfied, one-way ANOVA test followed by Student-Newman-Keuls test was used to assess differences among groups. If one or both conditions was/were not satisfied, Kruskal-Wallis ANOVA was used followed by Tukey’s test. The significance level was set at 5%.

**RESULTS**

The frequency distribution of particle diameters (Fig. 1) shows that our DEP contains 90% of particles with a diameter below 22.60 μm, followed by 50% of particles below 6.80 μm and also 10% below 1.54 μm. The average sizes of the particles were 10.02 and 3.60 μm according to their volume and surface, respectively. DEP metal and organic contents of this particulate matter were previously reported (13).

Est, P2, Ptot, and E increased in DIE group (Fig. 2) in relation to CTRL, EUG, and DEUG that did not differ among them. DIE also had increased PMN infiltration and alveolar collapse (Figs. 3 and 4). Thus eugenol avoided the impairment of lung tissue mechanical parameters, lung structure, as well as inflammation.

TUNEL and cleaved caspase-3 provided similar results, as shown in Fig. 5. DIE animals presented the highest amount of

![Fig. 1. Histogram of the frequency distribution of particle diameters (columns) and accumulated frequency (solid line).](image1)

![Fig. 2. Lung mechanics in mice. A: static elastance (Est) and viscoelastic component of elastance (ΔE) are shown. B: resistive (ΔP1), viscoelastic (ΔP2), and total pressures (ΔPtot) are depicted. DIE group significantly increased Est and ΔE (A), ΔP2, and ΔPtot (B) in relation to CTRL, EUG, and DEUG that did not differ among them. CTRL, DIE, EUG, and DEUG animals received saline, diesel particles, saline plus eugenol, and diesel particles plus eugenol, respectively. All animals were analyzed 24 h after gavage. Values are means ± SD of 10 mice per group. The differences between the groups are indicated by horizontal lines, over which the respective P values can be found.](image2)

![Fig. 3. Alveolar collapse (A) and influx of polymorpho- (PMN) and mononuclear (MN) cells (B). DIE group significantly increased alveolar collapse (A) and PMN infiltration (B) in relation to CTRL, EUG, and DEUG that did not differ among them. CTRL, DIE, EUG, and DEUG animals received saline, diesel particles, saline plus eugenol, and diesel particles plus eugenol, respectively. Values are means ± SD of 10 mice per group measured 24 h after gavage. The differences between the groups are indicated by horizontal lines, over which the respective P values can be found.](image3)
apoptosis that was completely or partially avoided by eugenol (according to TUNEL and caspase-3 techniques, respectively; DEUG group). Interestingly, TUNEL technique resulted in a lower value in EUG mice than in the other groups.

No beneficial effects on lipid peroxidation (oxidative stress marker) were observed in DEUG group. DIE and DEUG showed a higher level of MDA than EUG; additionally, the latter presented the lowest amount of MDA (Fig. 6). This figure also depicts a significantly smaller value of CAT in EUG animals than in the other three groups that did not differ among them.

**DISCUSSION**

This study aimed to investigate the putative protective role of eugenol against pulmonary damage caused by environmentally relevant doses of intact diesel particles. For such purpose, lung mechanics, architecture, as well oxidative stress, apoptosis, and inflammatory cells influx in lung parenchyma were used as markers.

We sought to expand the knowledge on the therapeutic use of eugenol, approaching its oral administration in mice exposed to air pollution. The choice of this route of administration stemmed from the fact that, although the peritoneal cavity represents a significant absorptive surface and the intraperitoneal injection is a common laboratory procedure, the latter is rarely used in the clinical practice. Therefore, to get closer to clinical procedures, we opted for the oral administration of eugenol.

Ambient particles have been consistently associated with adverse health effects, yielding to high cardiopulmonary morbidity and mortality (14, 22, 32). Particulate matter (PM) is a heterogeneous mixture of gas, liquid, and solid particles of different origins and sizes in suspension in the air, keeping intimous physical and chemical interactions. PM is classified, according to its aerodynamic diameter, as coarse (2.5–10 μm; PM10), fine (0.1–2.5 μm; PM2.5), and ultrafine (≤0.1 μm) (6). Epidemiological studies have demonstrated a strong association between particulate matter and lung diseases like asthma and chronic obstructive pulmonary disease (14, 22). Additionally, experimental studies provide evidence that lung inflammation, fibrosis, cancer, and systemic cardiovascular diseases are correlated with exposure to these particles (6). The different profiles of size and composition may influence particle toxicity and, consequently, the magnitude of adverse health effects (32). The special size and surface properties are the driving features leading to these effects. Indeed, as a consequence of the small size of these particles, they can easily penetrate and settle deep inside the lungs (14, 22). Our results showed that 90% of DEP were below 22.60 μm in diameter, being 50% below 6.80 μm and 10% below 1.54 μm (Fig. 1) . Thus at least an important fraction of the administered DEP reached the lung periphery. In human beings, toxicity becomes very important when aerodynamic diameter of the particles is <10 μm, which enables them to achieve the pulmonary alveoli. In rats and mice, this value approximates 2 μm for intratracheally instilled silica (39).

Our diesel metal and PAH contents, previously described (13), contain predominantly iron, sulfur, pyrene, benzo[a]anthracene and benzo[a]pyrene, but many other metals and PAHs were detected. Organic compounds, like PAH and quinone, which constitute ~30% of the weight of DEPs, have been reported to be potentially carcinogenic for humans (12, 22).
These organic compounds generate ROS in macrophages (12, 22). Additionally, PAH and quinone play a key role in the activation of SAPKs, which regulate the expression of pro-inflammatory genes in macrophages (12), thus suggesting that oxidative stress is one of the mechanisms by which DEPs exert their toxic effects in the lungs. DEPs may, independently or in association with other material, present many diverse effects, including the decrease in alveolar macrophage function (35), impairment of mucociliary clearance, modification of the surfactant composition (10) and secretion (19), among other deleterious effects. Laks and coworkers (13) reported that metals and sulfur components of diesel affected particle toxicity to a lesser extent than its organic compounds. The strongest associations between undesired health endpoints are observed for elements, such as Fe, Mn, Cu, S (40), and high-molecular-weight PAHs, when vehicular emissions are considered (40). Fe is importantly associated with the production of oxidative stress (27) by generating reactive oxygen species and facilitating superoxide anions ($O_2^-$) and hydrogen peroxide ($H_2O_2$) conversion to hydroxyl ions ($OH^-$) (27). The resultant pulmonary effects would include surfactant dysfunction (5), epithelial damage, increased vascular permeability, and inflammatory response, followed by impaired pulmonary function (8). Other elements, such as V, Zn, Cu, Ni, Co, and Cr, also present in our sample, can generate reactive oxygen species (to a lesser extent than Fe) that lead to inflammation and oxidative stress (34).

We used the same dose of DEP (15 μg) and mice weight (20–25 g) previously reported by our group (13). The same previously reported timeline was followed, i.e., the animals were studied 1 day after the administration of diesel particles. In this context, Nemmar and coworkers (23), using the same dose as us (15 μg/mice), administrated by intratracheal (it) instillation reported an exacerbation of thrombotic events by DEP in a mouse model of hypertension, providing an explanation for the cardiovascular morbidity and mortality accompanying urban air pollution. Another study (37) disclosed an alternation of spontaneous locomotor activity (SLA) and monoamine levels in the CNS of mice following in utero exposure of a low dose of DEP (171 μg DEP/m$^3$) for 8 h/day on gestational days 2–16. Higher doses of DEP are expected to impair the respiratory system as a result of particle overload. In this context, Sagai and coworkers (30) studied an “asthma-like” condition that develops in mice after prolonged intratracheal challenge with 100–200 μg of DEPs per week. Not only did these animals demonstrate an increase in airway resistance and mucus secretion, but there was also a marked infiltration of eosinophils in bronchi and medium-sized bronchioles, as well as a proliferation of goblet cells (30). Takano and coworkers (38) reported DEP effects on cytokine production in mice challenged intratracheally with 100-μg DEPs for 6 wk: IL-2, IL-4, IL-5, and GM-CSF levels were increased compared with control animals. Moreover, macrophage, eosinophil, neutrophil, and lymphocyte numbers were significantly increased in broncho-alveolar lavage fluid from animals challenged with DEPs plus antigen compared with animals treated with either agent alone.

Our results also showed that eugenol, administered 1 h after a single intranasal instillation of 15 μg of DEP, significantly..
improved lung static elastance, viscoelastic and total resistive pressures, and viscoelastic component of elastance (Fig. 2). It is well documented that pulmonary tissue mechanics are highly affected by lung parenchyma structure and composition (9). DEP administration induces changes in surfactant (5, 10, 19), favoring alveolar collapse (Figs. 3 and 4) leading to increased airway resistance, resulting in tissue and airway involvement correlated with viscoelastic parameters ($\Delta E$ and $\Delta P$). It should be pointed out that the power values of the ANOVAs concerning Est and $\Delta P$ were 0.62 and 0.70, respectively, and thus the results should be cautiously interpreted.

DEP also promoted significant morphological alterations in lung parenchyma. Our results showed higher PMN count and airspace collapse (Figs. 3 and 4) in DIE than in the other groups, which is in agreement with Laks and coworkers (13). Eugenol avoided these outcomes. The precise mechanisms of the anti-inflammatory action of eugenol are still disputed. A preventive effect of eugenol and other related compounds has been shown on LPS-induced NF-$\kappa$B activation, inflammatory cytokine release, and COX-2 expression in human and mouse macrophage cells stimulated in culture (20, 21). Apoptosis was higher in DIE than in the other mice (Fig. 5). Indeed, the lungs are constantly exposed to xenobiotics and PM that can damage them. The results from this study showed a significant increase in the number of apoptotic cells in animals exposed to DEPs. This result suggests that defense cells were absorbing DEPs in an effort to remove the xenobiotics from the lungs. DEP-exposed animals that were treated with eugenol presented a significantly smaller number of apoptotic cells than those in the DIE group (Fig. 5), indicating that eugenol helps to reduce cell death. Interestingly, eugenol was able to diminish apoptosis in CTRL mice, i.e., reduced the physiological rate of apoptosis.

In this study, we investigated the role of the phenol antioxidant, eugenol, in protecting the lungs from DEP-induced oxidative damage. DIE showed a higher level of the lipid peroxidation by-product MDA (Fig. 6A) than EUG, but the amount of CAT did not change in relation to CTRL (Fig. 6B). It should be pointed out that the power value of the ANOVA concerning CAT was 0.76, and thus the results should be cautiously interpreted. We expected that eugenol could protect lungs against DEP-induced oxidative damage; however, it was not able to return diesel-induced oxidative stress back to control levels, as evaluated by MDA. On the other hand, DEP did not modify CAT levels, so it is not surprising that DEUG and DIE presented similar values. In this context, other antioxidants also present opposing results: vitamins C and E have shown contradictory effects on oxidative stress-induced airway disorders (36); attempts to supplement the lung with reduced GSH itself or its precursors have also provided unconvincing results, e.g., nebulized GSH increases bronchial hyperreactivity as an additional side-effect (17); and administration of the amino acid cysteine, which is rate limiting for GSH synthesis, was also ineffective, since it was oxidized to neurotoxic cystine (18). However, positive results have been obtained using cysteine donors, N-acetylcysteine (NAC) or (L)-2-oxo-4-thiazolidine carboxylate (OTC), which have the potential to produce GSH. It is known that NAC increases plasma GSH levels dose dependently (4). Results of oxidative stress here have some limitations. Experimental design was very short, and specific methods for oxidative stress used here could be not sensitive for that. The dosage of DEP and/or EUG used may have been proportional to the intensity of markers or levels of detection. Mouse strain is directly and proportionally related to oxidative response (28).

This study presents limitations. We did not exhaustively study the mechanisms underlying oxidative stress and apoptosis. However, we aimed at treating the effects of exposure to an air pollutant with an agent that is both inexpensive and used world-wide in other conditions.

In conclusion, eugenol avoided changes in lung mechanics, pulmonary inflammation, and alveolar collapse elicited by diesel particles. It attenuated the activation signal of caspase-3 by DEP, and apoptosis evaluated by TUNEL was avoided. Finally, eugenol could not avoid oxidative stress as indicated by MDA.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s). Not applicable

AUTHOR CONTRIBUTIONS


REFERENCES


