Ambient particulate air pollution from vehicles promotes lipid peroxidation and inflammatory responses in rat lung

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Abstract

Oxidative stress plays a major role in the pathogenesis of particle-dependent lung injury. Ambient particle levels from vehicles have not been previously shown to cause oxidative stress to the lungs. The present study was conducted to a) determine whether short-term exposure to ambient levels of particulate air pollution from vehicles elicits inflammatory responses and lipid peroxidation in rat lungs, and b) determine if intermittent short-term exposures (every 4 days) induce some degree of tolerance. Three-month-old male Wistar rats were exposed to ambient particulate matter (PM) from vehicles (N = 30) for 6 or 20 continuous hours, or for intermittent (5 h) periods during 20 h for 4 consecutive days or to filtered air (PM <10 µm; N = 30). Rats continuously breathing polluted air for 20 h (P-20) showed a significant increase in the total number of leukocytes in bronchoalveolar lavage compared to control (C-20: 2.61 x 10⁵ ± 0.51; P-20: 5.01 x 10⁵ ± 0.81; P < 0.05) and in lipid peroxidation ([MDA] nmol/mg protein: C-20: 0.148 ± 0.01; P-20: 0.226 ± 0.02; P < 0.05). Shorter exposure (6 h) and intermittent 5-h exposures over a period of 4 days did not cause significant changes in leukocytes. Lipid damage resulting from 20-h exposure to particulate air pollution did not cause a significant increase in lung water content. These data suggest oxidative stress as one of the mechanisms responsible for the acute adverse respiratory effects of particles, and suggest that short-term inhalation of ambient particulate air pollution from street with high automobile traffic represents a biological hazard.

Key words

• Oxidative stress
• Reactive oxygen species
• Lipid peroxidation
• Particulate air pollution
• Lung inflammation
• Acute effects

Introduction

Epidemiological studies have shown that exposure to particulate matter is associated with increased cardiopulmonary morbidity and mortality (1-9), hospital admissions (10,11), and sudden death (12). However, the mechanism by which particulate matter promotes these effects is not understood. Stringer and Kobzik (13) suggested that particulate matter toxicity might be due to increased generation of reactive oxygen spe-
cies in target cells. Gurgueira et al. (14) reported that short-term exposure to concentrated ambient particles (CAPs) leads to a significant increase in oxidant levels in the heart and lung of rats which was associated with edema in these tissues. Rhoden et al. (15) showed that previous treatment with a general antioxidant (N-acetylcysteine) prevented lung inflammation induced by short-term CAP exposure.

The data cited above suggest that oxidative stress to the pulmonary parenchyma plays a major role in the pathogenesis of particle-dependent lung injury. However, it is important to note that the oxidative stress induced by particles in experimental animal studies was obtained with concentrated ambient particles or with tracheal instillation (14,15). Particles in the ambient from vehicles have not been shown to cause oxidative stress to the lungs. The present study was conducted in order to address this question, with two main objectives: a) to determine whether short-term exposure (6 or 20 h) to ambient levels of urban aerosols from a street with high automobile traffic elicits oxidative damage to membrane lipids in the lung, and b) to determine if intermittent short-term exposures (5 h per day) every 4 days induce some degree of tolerance.

Material and Methods

Site of exposure

Porto Alegre has a population of approximately 1.5 million inhabitants; it is the central city of an industrialized metropolitan area of 3.2 million inhabitants. The vehicle fleet was 522,555 vehicles in March 2004 (16), or about 1 vehicle for every 2.7 individuals, and an increase of 4.5% vehicles occurs every year. Vehicle traffic is the main source of air pollution in the larger cities of Brazil, being responsible for approximately 90% of carbon monoxide and particulate matter\textsubscript{10} emissions, with air pollution representing a serious problem in many regions (17,18). The total area of Porto Alegre is 496,827 km\textsuperscript{2} with 40% of the urban area being occupied by the transportation structure (16). This city presents frequent thermal inversions and defined climate seasons. Average temperature ranges from 18 to 25°C, and average rainfall days range from 3 to 6 per month (based on 8 years of historical weather readings; climate zone http://www.climate-zone.com/climate/brazil/fahrenheit/porto-alegre.htm).

Animals

Adult 3-month-old male Wistar rats from the Animal Facility of Fundação Faculdade Federal de Ciências Médicas de Porto Alegre were used. Animals were kept under 12-h light-dark periods and had free access to a standard laboratory diet (Supra-lab, Alisul Alimentos S/A., São Leopoldo, RS, Brazil) and water before and during the exposure.

Exposure chambers

Exposure chambers were made of glass (30 x 30 x 50 cm) and were hermetically closed, with the exception of one entrance (connected to the outdoor environment) and one exit. A vacuum pump was connected to the exit and pumped outdoor air through the exposure chambers at a flow rate of 10 L/min. Inhalation chambers were located on the second floor of the building of Fundação Faculdade Federal de Ciências Médicas de Porto Alegre, located in downtown Porto Alegre at a crossroad with heavy traffic (Sarmento Leite Street and André da Rocha Street), facing an automated air pollution monitoring station of the State Environmental Agency, that provides continuous particulate matter measurements (beta monitor). One of the chambers (designated as control) was equipped with a 37-mm Teflon filter (Millipore, Carrigtwohill, Ireland) in the inlet, which prevented the admission
of ambient particles, whereas the other received atmospheric air without the filtering system (designated as polluted). The temperature in the room and in the chamber was 22 ± 2°C.

Experimental protocol

Continuous short-term exposure to particulate matter. Rats were exposed to ambient particles from vehicles (polluted group) or filtered air (control group) for periods of 6 h (on August 4, 2004) or 20 h (on August 17, 2004), as described above. The animals were allowed to move freely in the cage during the exposure and both groups were exposed and tested simultaneously. Immediately after the exposure, animals were anesthetized ip with 50 mg/kg pentobarbital and killed by exsanguination. The right lung of each animal from both groups was excised and used to determine lung edema. Another group of animals was submitted to the same protocol and to bronchoalveolar lavage 24 h later to determine the inflammatory parameters. The protocol of bronchoalveolar lavage for the 6-h exposure was performed on two different days (July 12 and July 20, 2004), whereas the 20-h exposure was performed on September 17, 2004. The ambient levels of particulate matter concentrations on the days of exposure are listed in Table 1. The animals were awake and movement was unrestricted during exposure. Both groups were exposed and tested simultaneously. Immediately after the last exposure the animals were submitted to the same procedure as described above for the collection of lung samples for lipid peroxidation and edema determination.

Tissue preparation

The lungs were excised, washed in saline solution and quickly frozen in liquid nitrogen. For the determination of thiobarbituric acid reactive substances (TBARS), tissue samples were homogenized in 5 volumes of 120 mM KCl and 30 mM sodium phosphate buffer, pH 7.4, containing 0.5 mM phenylmethanesulfonyl fluoride as a protease inhibitor, at 0-4°C. The suspensions were centrifuged at 600 g for 10 min at 0-4°C to remove nuclei and cell debris. The pellets were discarded and the supernatant solutions were used as homogenates.

Determination of thiobarbituric acid reactive substances - lipid peroxidation

Lung homogenates were precipitated with 10% TCA, centrifuged, and incubated with thiobarbituric acid (Sigma, St. Louis, MO, USA) for 15 min at 100°C and TBARS were

<table>
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<th>Protocol</th>
<th>Date</th>
<th>PM$_{10}$ (µg/m$^3$)</th>
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<tr>
<td>Continuous short-term exposure</td>
<td>July 12, 2004</td>
<td>34.0</td>
</tr>
<tr>
<td>(6 h)</td>
<td>July 20, 2004</td>
<td>22.0</td>
</tr>
<tr>
<td>Continuous short-term exposure</td>
<td>August 4, 2004</td>
<td>224.7</td>
</tr>
<tr>
<td>(6 h)</td>
<td>August 17, 2004</td>
<td>138.6</td>
</tr>
<tr>
<td>Continuous short-term exposure</td>
<td>September 17, 2004</td>
<td>112.4</td>
</tr>
<tr>
<td>(20 h)</td>
<td>September 6-9, 2004</td>
<td>99.2</td>
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</table>
extracted with butanol (1:1 v/v). After centrifugation, the absorbance of the butanol was measured at 535 nm (20). The amount of TBARS formed is reported as nmol/mg protein. Malondialdehyde standards were prepared from 1,1,3,3,-tetramethoxypropane. Protein concentration in the homogenates was measured by the Bradford protein assay (21) using bovine serum albumin as standard. Measurements were carried out in a Perkin Elmer Lambda 40 spectrophotometer (Shelton, CT, USA).

Lung edema

The severity of pulmonary edema was determined by the wet/dry ratio. Lung samples (about 200 mg) taken from the same animals as used for TBARS determination were weighed and dried in a conventional oven (at 90°C) until they reached constant weight. The results are reported as wet/dry ratios (g/g).

Bronchoalveolar lavage

Rats exposed to polluted or filtered air were anesthetized with sodium pentobarbital (50 mg/kg body weight) 24 h after exposure and euthanized by exsanguination. The trachea was sectioned, a fine catheter was introduced and fixed with cotton thread, and 21 mL sterile saline solution was injected in three series of 7 mL, followed by aspiration. The rate of recovery of the injected solution was approximately 80%. Each aliquot represents one injected and one drawn in recovery of fluid. The recovered fluid was centrifuged at 400 g at 6°C and the supernatant from the first lavage was saved for the measurement of protein level. Total cell counts were determined after Trypan blue staining using a Neubauer chamber. Total protein levels, as a measure of vascular permeability, were determined in the supernatant of the first lavage aliquot by the Bradford protein assay (21) using a Perkin Elmer Lambda 40 spectrophotometer.

Animal care

Animals were handled humanely throughout the study to minimize their discomfort and to prevent distress. All protocols were approved by the Research Ethics Committee of Fundação Faculdade Federal de Ciências Médicas de Porto Alegre (CPA 085/03).

Statistical analysis

Data are reported as means ± SEM. Statistical analyses were performed by the unpaired Student t-test using the Sigma-Stat 2.0 Software (Jandel Corporation, 1992-1995, San Jose, CA, USA), with the level of significance set at 5%.

Results

Rats breathing ambient particulate matter from vehicles for 6 h did not show increased lipid peroxidation in the lungs (Figure 1A). However, when the animals were continuously exposed for 20 h a statistically significant increase in this parameter was detected in their lungs (Figure 1B), but not in the lungs of rats exposed intermittently to particulate matter for 20 h (Figure 1C).

Rats exposed to filtered air or not for 6 h did not show an increase in leukocytes in bronchoalveolar lavage (BAL; Figure 2A). In agreement with the results of lipid peroxidation, rats continuously exposed to vehicular pollution for 20 h showed a significant accumulation of leukocytes in BAL, as compared with BAL from rats exposed to filtered air (Figure 2B). However, accumulation of leukocytes in BAL was not accompanied by significant changes in total protein levels (continuous short-term 6 h: control = 0.96 ± 0.10 vs polluted = 1.05 ± 0.08 mg/mL, P = 0.607; continuous short-term 20 h: control = 1.34 ± 0.14 vs polluted = 1.39 ± 0.09 mg/mL,
P = 0.763). Finally, the wet/dry ratio, a measurement of global tissue damage, was not changed by exposure to pollution (continuous short-term 6 h: control = 4.87 ± 0.09 vs polluted = 4.85 ± 0.18 g/g, P = 0.924; continuous short-term 20 h: control = 5.25 ± 0.08 vs polluted = 5.20 ± 0.09 g/g, P = 0.688; intermittent short-term - total 20 h: control = 4.86 ± 0.13 vs polluted = 5.20 ± 0.18 g/g, P = 0.167).

Discussion

In the present study, rats were exposed to vehicle polluted (urban) or clean (filtered) ambient air on a short-term basis. Their lungs were evaluated for lipid damage and showed a time-dependent increase of lipid peroxidation. Rats exposed to particulate matter for 20 h presented an almost 2-fold higher lipid peroxidation when compared to rats exposed for 6 h. Our results agree with previous studies with animals breathing CAPs for 5 h, which presented an increase in oxidant levels in the lung and heart (14,19). Both observations would be compatible with Fenton-type reactions catalyzed by transition metals, redox-cycling processes, or biochemical changes triggered by non-covalent binding to membrane receptors. It is also important to point out that particulate matter concentrations in studies using the CAP model were 300-400 µg/m³ (total mass - on average) while in our study they were 110-140 µg/m³. In addition, our findings showed that even a lower concentration of particulate matter, considered safe, was able to promote damage to membrane lipid in the lung.

Pro-inflammatory and toxic effects of particulate matter have been reproduced in the laboratory in studies on humans (22), on animal models (23,24) and on cells in culture (25). Pulmonary oxidative stress is related to polymorphonuclear neutrophil (PMN) count in BAL and to PMN infiltration (26-29), and these biological effects were prevented by N-acetylcysteine treatment (15). We observed that rats exposed to ambient vehicular pollution for 20 h in Porto Alegre showed a significant accumulation of PMN leukocytes in BAL (Figure 2B), a fact that was not detected in rats submitted to 6 h of exposure (Figure 2A). PMN influx was not accompanied by significant changes in total protein levels, in agreement with the findings of Rhoden et al. (15). Particulate matter-induced oxidative stress is associ-
ated with the development of lung inflammation and tissue damage (27,30,31). However, the wet/dry ratio, a measurement of global tissue damage, did not show a significant increase as a function of exposure time in the present study, in contrast to results reported by other investigators that showed significant development of lung edema in animals exposed to CAPs for 5 h (14). One limitation of the present study was that we did not determine the composition of particulate matter. Particularly important in this respect is the fact that experimental studies with CAPs have been performed with higher doses of particulate matter, resulting in different levels of lung inflammatory effects. However, our experimental design had the advantage of reproducing the real concentration of particulate matter for which effects have been detected in epidemiological studies (1-9) and the results observed offer important evidence regarding inflammation and the oxidant effects of particulate matter at sites with acceptable pollution indexes.

The experimental model employed in this study indicates that combining animal studies with “real world exposures” may be of use to understand the pathogenesis of lung injury promoted by urban particles, as well as to devise strategies of pollution control for the minimization of health effects.

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References