Human SLPI inactivation after cigarette smoke exposure in a new in vivo model of pulmonary oxidative stress

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Cavarra, Eleonora, Monica Lucattelli, Federica Gambelli, Barbara Bartalesi, Silvia Fineschi, Andras Szarka, Fabiola Giannerini, Piero A. Martorana, and Giuseppe Lungarella. Human SLPI inactivation after cigarette smoke exposure in a new in vivo model of pulmonary oxidative stress. Am J Physiol Lung Cell Mol Physiol 281: L412–L417, 2001.—The role of oxidative stress in inactivating antiproteases is the object of debate. To address this question, we developed an in vivo model of pulmonary oxidative stress induced by cigarette smoke (CS) in mice. The major mouse trypsin inhibitor contraspins is not sensitive to oxidation, and the mouse secretory leukoprotease inhibitor (SLPI) does not inhibit trypsin. Instead, human recombinant (hr) SLPI inhibits trypsin and is sensitive to oxidation. Thus we determined the effect of CS in vivo on hrSLPI antiproteolytic function in the airways of mice. CS caused a significant decrease in total antioxidant capacity in bronchoalveolar lavage fluid (BALF) and significant changes in oxidized glutathione, ascorbic acid, protein thiols, and 8-epi-PGF2α. Intratracheal hrSLPI significantly increased BALF antiproteolytic activity. CS induced a 50% drop in the inhibitory activity of hrSLPI. Pretreatment with N-acetylcysteine prevented the CS-induced loss of hrSLPI activity, the decrease in antioxidant defenses, and the elevation of 8-epi-PGF2α. Thus an inactivation of hrSLPI was demonstrated in this model. This is a novel model for studying in vivo the effects of oxidative stress on human protease inhibitors with antiproteolytic activity.

antioxidant status; secretory leukoprotease inhibitor inactivation; bronchoalveolar lavage

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) is a serious disorder that accounts for much human suffering and is currently the fourth leading cause of death in the US (1). Cigarette smoke is the most prominent factor determining the increased prevalence and mortality of COPD worldwide (2). Also, epidemiologic studies have established that centrilobular emphysema, which is a major component of the morbidity and mortality of COPD, is associated with the cigarette smoking habit (13). The prevalent hypothesis to explain the lung destruction in emphysema is that smoking causes a proteolytic imbalance in the peripheral lung that is responsible for parenchymal destruction (31). This imbalance has been attributed to both an increased number of inflammatory cells, which can release proteolytic enzymes capable of destroying lung tissue (i.e., elastases), and a functional deficit of antiproteases due to oxidation of their active site.

There are numerous reports (for a review, see Ref. 26) of increased oxidative stress in smokers and in patients with COPD, and several mechanisms have been postulated by which oxidants can cause lung dysfunction. It has been postulated that an imbalance in favor of oxidants can lead to lung injury either directly via oxidative damage to alveolar epithelial cells and extracellular matrix components or indirectly via oxidative inactivation of antiproteases [i.e., α1-protease inhibitor (α1-PI) and secretory leukoprotease inhibitor (SLPI)]. This would result in an enhanced proteolysis of lung connective components such as elastin, which is a key event in the pathogenesis of emphysema (31).

The ability of an increased oxidant burden to cause lung dysfunction and injury is dependent, in part, on the balance between oxidants and antioxidants. Unfortunately, there is limited information on the lung antioxidant defenses in smokers and even less in COPD patients, and there is no clear evidence “in vivo” that cigarette smoke alters the antioxidant defenses with inactivation of antiprotease molecules. This fact may be due to the obvious difficulty in studying, under in vivo conditions, an “open system” such as the antioxidant defense system, present both extra- and intracellularly, and also to the difficulties encountered in the choice and measurement of specific markers of oxidative injury.

To address this question, we specifically designed a study to develop an experimental animal model of oxidative stress induced by cigarette smoke where we could investigate the oxidative inactivation of lung
protease inhibitors with antitryptic activity (i.e., SLPI and α₁-PI). The observation that mouse SLPI does not have antitryptic activity and that the major mouse antitrypsin contraspin is not sensitive to oxidation (35) allowed the development of such an in vivo model in this animal. Under our experimental conditions, cigarette smoke induces a mild oxidative stress characterized by a transient but significant decrease in lung antioxidant defenses and reduced the antitryptic activity of exogenous human recombinant (hr) SLPI by ~50%. Pretreatment with N-acetylcysteine (NAC) prevented the loss of hrSLPI activity. This model represents a new tool for studying in vivo the inactivation of protease inhibitors with antitryptic activity and the efficacy of antioxidant molecules of potential therapeutic importance.

**MATERIALS AND METHODS**

**Animals and treatments.** Male C57BL/6J mice (supplied by Charles River, Calco, Italy) 3–4 mo of age were used in this study. The mice were housed in groups of two to four in macrolon cages (room temperature, 22–24°C; relative humidity, 40–50%; food and water supplied ad libitum). All animal experimentation was conducted in conformity with the Guiding Principles for Research Involving Animals and Human Beings and was approved by the Local Ethical Committee of the University of Siena (Siena, Italy).

The animals were divided into 3 groups of 35 animals each: 1) a control group that was not subjected to any kind of manipulation, 2) a group that was exposed to cigarette smoke, and 3) a sham-exposed group exposed to room air in the smoking machine under the same conditions as group 2.

In another experiment, an additional 10 animals from groups 1 and 2 were treated with hrSLPI and/or NAC. hrSLPI (a gift from Dr. Jan Stolk, University Medical Centre, Leiden, The Netherlands) was given intratracheally under light ether anesthesia at a dose of 235 µg in saline solution. NAC (Sigma, St. Louis, MO) dissolved in water was administered orally 4 h before smoke exposure at a dose of 1 g/kg body wt. The dose of NAC was based on doses previously employed by others in similar studies (14, 29, 30).

**Exposure to cigarette smoke.** Mice were exposed for 20 min to either smoke from five cigarettes (commercial Virginia cigarettes; 12 mg of tar and 0.9 mg of nicotine) or room air in especially designed macrolon cages (Tecniplast, Buguggiate, Italy), essentially according to Escolar et al. (9). These cages (42.5 × 26.6 × 19 cm; 21.47 dm³) were equipped with a disposable filter cover with fifteen 10-mm holes that enable the air to flow out of the cages and thus be continuously renewed. The smoke was produced by burning a cigarette and was introduced into the chambers with the airflow generated by a mechanical ventilator (7025 rodent ventilator, Ugo Basile, Biological Research Instruments, Comerio, Italy) at a rate of 250 ml/min. A second mechanical ventilator was used to provide room air for dilution (1:10) of the smoke stream. In a pilot study, the efficiency of the smoke delivery system was tested by measuring blood carboxyhemoglobin (COHb) in 12 mice by CO oximetry.

Bronchoalveolar lavage and blood samples. Immediately after smoking, blood samples were drawn from the right ventricle of the animals under light ether anesthesia. The trachea was isolated and then cannulated with a 20-gauge blunt needle. With the aid of a peristaltic pump (P-1 Pharmacia), the lungs were lavaged in situ three times with 0.6 ml of saline solution. The average fluid recovery was >95%.

These procedures were also done in additional groups of 10 animals each 20 and 60 min after smoke exposure.

Total cell counts were performed with a hemacytometer on bronchoalveolar lavage fluid (BALF). Differential counts of 300 cells were done on slides stained with Diff-Quik.

Cell-free BALF obtained after centrifugation at 600 g for 15 min was used for biochemical assays. Total protein concentration was determined in cell-free BALF according to Lowry et al. (17). Blood samples were assayed for COHb by CO oximetry.

**Antioxidant status in BALF.** Total antioxidant capacity was measured in cell-free BALF according to Miller et al. (19). The antioxidant capacity of BALF was then compared with Trolox as “Trolox equivalent antioxidant capacity” (TEAC). The TEAC in BALF samples is equal to the concentration of a Trolox solution having the same antioxidant capacity. This is expressed in micromoles per milliliter (19).

The impact of cigarette smoke on the redox status of the respiratory tract lining fluids was also examined by determining water-soluble (ascorbate, thiols) and lipid-soluble (vitamin E) antioxidants in cell-free BALF.

Total and oxidized (GSSG) glutathione were assayed in cell-free BALF with the enzyme recycling method essentially as described by Tietze (33) with the following modifications. The thiol-scavenging reagent 1-methyl-2-vinylpyridinium trifluoromethanesulfonate was used instead of N-ethylmaleimide (BIOXYTECH GSH/GSSG-412, OXIS Health Products, Portland, OR).

Total thiols were measured with a spectrophotometric method with Ellman’s reagent, dithionitrobenzene (DTNB), according to Habeeb (10) with the following modifications. An aliquot of BALF (600 µl) was added to 150 µl of a solution containing 10% sodium dodecyl sulfate, 0.4 M sodium phosphate buffer, pH 8.0, and 0.5 mg/ml of EDTA. DTNB solution [25 µl of DTNB (0.4% wt/vol) in 0.1 M sodium phosphate buffer, pH 8.0] was added, and the color was developed for 15 min. Absorbance at 410 nm was determined against an appropriate blank. For calculation of sulphydryl content, the net absorbance was employed, with a molar extinction coefficient of 13,600 M⁻¹cm⁻¹. The protein thiol value was obtained by subtracting total glutathione from the total thiol value.

Ascorbic acid was determined in cell-free BALF according to the spectrophotometric method of Zannoni et al. (34), which relies on the reduction of ferric iron by ascorbic acid followed by formation of a complex of the ferrous iron product and α,α′-dipyridyl.

Vitamin E was extracted from cell-free BALF with a mixture of 75 mM sodium dodecyl sulfate solution-ethanol-n-heptane (1:2:1 vol/vol) according to Burton et al. (4) and measured by HPLC. The HPLC analysis was done with a 300 × 39-mm µBondapack C18 column. Vitamin E was separated in a mobile phase consisting of methanol-hexane-acetonitrile (60:20:20 vol/vol) at a flow rate of 1.0 ml/min and detected by ultraviolet absorbance at 290 nm (15).

8-epi-PGF₂α. The 8-epi-PGF₂α concentration was determined in plasma and in cell-free BALF with a commercial competitive enzyme-linked immunosassay kit (OXIS International).

**Trypsin inhibitory capacity in BALF.** BALF was tested for trypsin inhibitory capacity (TIC) against bovine pancreatic trypsin (BPT) (Sigma) according to Briscoe et al. (3), with N-α-benzoyl-l-arginine p-nitroanilide as the chromogenic substrate. BPT was active-site titrated (6) and found to be 76% active. Briefly, BALF samples (12.5 µl) and BPT (5.8 µM) were preincubated for 30 min at 25°C followed by the addition of substrate. The formation of p-nitroanilide was detected by ultraviolet absorbance at 290 nm (15).
followed spectrophotometrically at 410 nm over a 5-min period at 25°C. TIC is expressed as micrograms of trypsin inhibited per milliliter of BALF.

Statistical analysis. Intergroup comparison was made with one-way ANOVA (F-test). A \( P \) value of <0.05 was considered significant.

RESULTS

Smoking mouse as a model of mild oxidative stress. The exposure of mice to cigarette smoke resulted in a transient but significant decrease in the antioxidant capacity measured as TEAC in BALF (0.48 ± 0.07 vs. 0.67 ± 0.05 \( \mu \)mol/ml in unexposed control mice). This change was no longer present 20 min after smoke exposure.

A significant increase in GSSG was observed after smoking (0.73 ± 0.05 vs. 0.54 ± 0.03 \( \mu \)mol/mg protein in unexposed control mice). Ascorbic acid (80.1 ± 9.5 \( \mu \)g/mg protein) and protein thiols (3.3 ± 0.6 \( \mu \)mol/mg protein) were significantly less than the control values, which were 144.7 ± 10.1 \( \mu \)g/mg protein and 5.3 ± 0.5 \( \mu \)mol/mg protein, respectively. The mean values for total glutathione (17.38 ± 2.61 \( \mu \)mol/mg protein) and vitamin E (8.12 ± 2.45 \( \mu \)mol/mg protein) in animals exposed to cigarette smoke did not differ significantly from those observed in control animals (total glutathione, 19.44 ± 3.53 \( \mu \)mol/mg protein; vitamin E, 5.82 ± 2.30 \( \mu \)mol/mg protein). The percent changes in the values of TEAC and of both the water-soluble and lipid-soluble antioxidants after smoke exposure are reported in Fig. 1.

No differences in TEAC and in both water-soluble and lipid-soluble antioxidants in BALF have been found between sham-exposed and unexposed groups.

A consistent elevation in 8-epi-PGF\(_{2\alpha}\), a marker of oxidative stress, was observed in the BALF of mice exposed to cigarette smoke (unexposed control mice, 0.33 ± 0.03 ng/ml; smoking mice, 0.51 ± 0.05 ng/ml). A significant increase in 8-epi-PGF\(_{2\alpha}\) was also seen in the plasma of smoking mice (10.58 ± 1.1 ng/ml; \( n = 18 \)) with respect to that in the control group (8.81 ± 0.9 ng/ml; \( P < 0.05; n = 16 \)). Similar figures have been detected in mice 20 and 60 min after smoke exposure.

NAC treatment 4 h after its administration was able to significantly increase the baseline values of TEAC (0.97 ± 0.12 \( \mu \)mol/ml) and total glutathione (31.26 ± 1.98 \( \mu \)mol/mg protein) in BALF (Fig. 2) and to prevent the changes detected in TEAC, ascorbate, and 8-epi-PGF\(_{2\alpha}\) levels in smoking animals (Fig. 3). The mean values of TEAC, ascorbate, and 8-epi-PGF\(_{2\alpha}\) levels in the BALF of smoking animals treated with NAC were

![Fig. 1. Antioxidant status determined immediately in bronchoalveolar lavage fluid (BALF) samples after cigarette smoke exposure. Results are means expressed as percent of values from unexposed control mice (TEAC, 0.67 ± 0.05 \( \mu \)mol/ml; total glutathione, 19.44 ± 3.53 \( \mu \)mol/mg protein; oxidized glutathione (GSSG), 0.54 ± 0.03 \( \mu \)mol/mg protein; protein thiols, 5.3 ± 0.5 \( \mu \)mol/mg protein; ascorbic acid, 144.7 ± 10.1 \( \mu \)g/mg protein; vitamin E, 5.82 ± 2.30 \( \mu \)mol/mg protein); \( n = 16 \) mice. * \( P < 0.05 \) vs. control.](http://ajplung.physiology.org/content/281/4/L414.f1)

![Fig. 2. TEAC and total glutathione levels in BALF samples after N-acetylcysteine (NAC) administration. Results are means expressed as percent of values from untreated control mice (TEAC, 0.67 ± 0.05 \( \mu \)mol/ml; total glutathione, 19.44 ± 3.53 \( \mu \)mol/mg protein); \( n = 16 \) mice. * \( P < 0.05 \) vs. control.](http://ajplung.physiology.org/content/281/4/L414.f2)

![Fig. 3. TEAC, GSSG, ascorbic acid, and 8-epi-PGF\(_{2\alpha}\) levels in BALF samples after cigarette smoke exposure in animals pretreated with NAC. Results are means expressed as percent of values from unexposed control mice (TEAC, 0.67 ± 0.05 \( \mu \)mol/ml; GSSG, 0.54 ± 0.03 \( \mu \)mol/mg protein; ascorbic acid, 144.7 ± 10.1 \( \mu \)g/mg protein; 8-epi-PGF\(_{2\alpha}\), 0.33 ± 0.03 ng/ml); \( n = 16 \) mice. * \( P < 0.05 \) vs. control.](http://ajplung.physiology.org/content/281/4/L414.f3)
0.68 ± 0.16 μmol/ml, 130.7 ± 30.2 μg/mg protein, and 0.30 ± 0.04 ng/ml, respectively. No change in GSSG level was observed after NAC treatment (0.52 ± 0.05 nmol/mg protein).

It is of interest that the blood COHb level increased from an undetectable baseline level (determined in unexposed control animals) to 14.69 ± 3.39% in the smoke-exposed group. No substantial change in COHb levels was observed in blood of the sham-exposed group (0.1 ± 0.1%).

With regard to the total and differential cell counts and protein content in BALF, no differences were observed among the various experimental groups (Table 1).

**Smoking mouse as a model in which to study human trypsin inhibitors.** After smoke exposure, the endogenous levels of TIC in BALF remained unchanged (123 ± 4 and 120 ± 5 μg trypsin inhibited/ml BALF for control and smoking mice, respectively). This makes the mouse acutely exposed to cigarette smoke a suitable model in which to study in vivo the effect of oxidation on the antitryptic activity of human protease inhibitors.

**Decrease in hrSLPI activity after smoke exposure.** The intratracheal administration of hrSLPI in mice resulted in a significant increase in TIC (95%) in BALF. As seen in Fig. 4, the mean TIC levels related to the activity of administered hrSLPI remained at high values within the first 3 h after instillation and decreased thereafter. In mice treated with hrSLPI 90 min before smoke exposure, cigarette smoke induced an ~50% drop in the inhibitory activity related to hrSLPI (Fig. 5).

NAC prevents the decrease in hrSLPI activity. We tried to prevent the decrease in TIC observed after smoke exposure by pretreating the mice with NAC administered orally 4 h before cigarette smoke exposure. As seen in Fig. 5, NAC administration at a dose of 1 g/kg body wt totally prevented the decrease in TIC induced by cigarette smoke in mice.

**DISCUSSION**

Centrilobular emphysema is a major component of the morbidity and mortality of COPD and is associated with the cigarette smoking habit (13). Several possible factors for the development of emphysema from exposure to cigarette smoke have been proposed. Among them, inflammation, proteases, and oxidants are considered to be the major contributors to the development of this disorder. In particular, the proteolytic burden that is thought to be responsible for parenchymal destruction in smokers is currently attributed to both an increased release of proteases by inflammatory cells and an oxidative inactivation of protease inhibitors due to an oxidant-antioxidant imbalance (24, 27, 32). How-

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Total Cells, 10⁵</th>
<th>Macrophages, %</th>
<th>Neutrophils, %</th>
<th>Lymphocytes, %</th>
<th>Protein Content, mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.55 ± 0.21</td>
<td>89.6 ± 2.35</td>
<td>5.2 ± 1.34</td>
<td>5.2 ± 1.49</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>Sham exposed</td>
<td>1.49 ± 0.16</td>
<td>89.5 ± 1.98</td>
<td>5.3 ± 1.42</td>
<td>5.2 ± 1.48</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>Cigarette smoke</td>
<td>1.34 ± 0.14</td>
<td>88.4 ± 1.58</td>
<td>5.9 ± 1.66</td>
<td>5.7 ± 1.53</td>
<td>0.39 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 5 animals in control and sham-exposed groups and 7 animals in cigarette smoke group. BALF, bronchoalveolar lavage fluid.
ever, evidence of the interaction between these mechanisms in the pathogenesis of smoking-induced lung diseases is still fragmentary. Clinical studies of the oxidant-antioxidant balance have focused on the chronic effect of smoking, whereas the effects of acute smoking have received less attention. Thus the pathogenic role of an oxidative stress under these conditions is still the object of debate (7).

The observation that, under our experimental conditions, the mouse lung antiprotease(s) with antitryptic activity is not sensitive to oxidation and that mouse SLPI, which is sensitive to oxidation, does not have antitryptic activity (35) allowed us to develop a novel in vivo model for the investigation of the oxidative inactivation of human antiproteases with antitryptic activity.

In this model, we provide in vivo evidence that acute exposure to cigarette smoke in the mouse lung induces 1) a mild oxidative stress characterized by a transient but significant drop in the antioxidant defenses and 2) a loss of activity of the human antiprotease SLPI, a significant component of the antielastase shield in the lung.

There is conflicting evidence for the presence of an antioxidant deficiency in the lungs of smokers or patients with COPD (26). This may depend on which antioxidant is studied and/or the phase of the inflammatory response that was investigated. With regard to the former point, the antioxidant defense system in the lung includes a number of antioxidants such as glutathione, ascorbic acid, vitamin E, albumin, and other enzymatic or nonenzymatic molecules, which all together are responsible for the antioxidant capacity of BALF (11, 28). This open system is in balance with other intra- and extracellular antioxidant defense systems (i.e., inflammatory cells and plasma) that are important for the maintenance of cellular function. The lung total antioxidant capacity measured by us as TEAC in BALF does mirror the combined activity of the various nonenzymatic and enzymatic antioxidants from both intra- and extracellular systems.

The results provided here demonstrate that acute smoke exposure in vivo can determine significant changes in BALF TEAC that can be ascribed to a depletion of a number of factors including protein sulfhydrys and ascorbic acid and to a perturbation in the glutathione redox system as revealed by the GSSG increase. There is now evidence that glutathione plays a critical role in maintaining epithelial integrity after exposure to cigarette smoke. In particular, it has been recently demonstrated that the increased epithelial permeability after exposure to cigarette smoke condensate is associated with profound changes in the homeostasis of the antioxidant glutathione (24). Further evidence for a central role played by glutathione derives from studies (16, 20, 25) demonstrating that depletion of lung reduced glutathione (GSH) alone by a specific glutathione synthesis inhibitor can induce an increased lung epithelial permeability “in vitro” and in vivo.

A study (5) in humans has shown that glutathione is elevated in the epithelial lining fluid of chronic cigarette smokers compared with nonsmokers. This increase did not occur during acute cigarette smoking in humans (21) and under our experimental conditions. The discrepancy between glutathione levels in chronic and acute cigarette smoking may find an explanation in the upregulation of important gene(s) involved in the synthesis of glutathione caused by oxidative stress (18). This event may act as a protective mechanism in chronic cigarette smokers. In particular, GSH may counteract the development of emphysema in chronic smokers by preventing smoke-related inactivation of SLPI and other antiproteases. This may be one of the reasons why only 15–20% of heavy smokers develop COPD.

Under our experimental conditions, acute smoke exposure can induce a transient oxidative imbalance that results in an oxidative injury as revealed by the increase in 8-epi-PGF2α, which has been considered to be a stable biomarker of free radical formation (8, 12, 22). In this milieu, even in absence of any detectable inflammatory reaction, a marked decrease in the activity of hrSLPI has been observed. Under our experimental conditions, the thiol-containing compound NAC is able to increase the antioxidant potential in BALF and to prevent the loss of hrSLPI activity. These effects may be due to the potential for NAC to act either directly as an antioxidant or indirectly, enhancing the levels of glutathione (26). Even though our data do not rule out a possible contribution of cigarette smoke aldehydes, they are strongly indicative of an important role played by free radical mechanisms in the inactivation of SLPI. Cigarette smoke, due to the high concentration of free radicals per puff (23), may cause transient but repeated oxidative imbalances, resulting in oxidative inactivation of antiproteases. This can render smokers particularly susceptible to the development of emphysema.

In summary, acute cigarette smoke exposure in the mouse represents a new and rapid in vivo model of pulmonary oxidative stress. This model is suitable for testing the in vivo efficacy of antioxidant molecules or for studying the pharmacokinetics of human protease inhibitors with antitryptic activity of potential therapeutic importance.

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