The tick-derived rBmTI-A protease inhibitor attenuates the histological and functional changes induced by cigarette smoke exposure

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Summary. Introduction. Smoking is the main risk factor for chronic obstructive pulmonary disease development and cigarette smoke (CS) exposure is considered an important approach to reproduce in rodents this human disease. We have previously shown that in an elastase-induced model of emphysema, the administration of a protease inhibitor (rBmTI-A) prevented and attenuated tissue destruction in mice. Thus, in this study we aimed to verify the effects of rBmTI-A administration on the physiopathological mechanisms of CS-induced emphysema. Methods. Mice (C57BL/6) were exposed to CS or room air for 12 weeks. In this period, 3 nasal instillations of rBmTI-A inhibitor or its vehicle were performed. After euthanasia, respiratory mechanics were evaluated and lungs removed for analysis of mean linear intercept, volume proportion of collagen and elastic fibers, density of polymorphonuclear cells, macrophages, and density of positive cells for MMP-12, MMP-9, TIMP-1 and gp91phox. Results. The rBmTI-A administration improved tissue elastance, decreased alveolar enlargement and collagen fibers accumulation to control levels and attenuated elastic fibers accumulation in animals exposed to CS. There was an increase of MMP-12, MMP-9 and macrophages in CS groups and the rBmTIA only decreased the number of MMP-12 positive cells. Also, we demonstrated an increase in gp91phox in CS treated group and in TIMP-1 levels in both rBmTI-A treated groups. Conclusion. In summary, the rBmTI-A administration attenuated emphysema development by an increase of gp91phox and TIMP-1, accompanied by a decrease in MMP-12 levels.

Key words: Protease inhibitor, Cigarette smoke, Emphysema, Metalloproteases, Animal models

Introduction

Although chronic obstructive pulmonary disease (COPD) is an important leading cause of death worldwide and is predicted to increase in prevalence over the next decades (GOLD, 2015), there are currently no effective pharmacotherapeutic strategies to inhibit COPD progression. The highest risk factors for COPD are tobacco smoking and outdoor, occupational and indoor air pollution (GOLD, 2015).

The majority of studies in COPD patients are restricted to lung samples obtained from pulmonary biopsies or resection. Therefore, to better understand the structural changes in lung parenchyma and airways during COPD development, animal models are often used to investigate the mechanisms involved in lung parenchyma destruction and remodeling (Mahadeva and Shapiro, 2002; Wright et al., 2008) and to test new therapeutic approaches (Kuraki et al., 2002; Wright et al., 2002; Churg et al., 2007). Most COPD models have
either used cigarette smoke (CS) or the association of CS and bacterial/viral administrations believed to reproduce some of the mechanisms behind the cigarette smoke (Fricker et al., 2014). Considering the imbalance between proteases and anti-proteases in emphysema development, the importance of MMPs in parenchymal fibers injuring have been attested (Shapiro, 1998; Belvisi and Bottomley, 2003; Churg et al., 2012; Robertoni et al., 2015). Hautamaki et al. (1997) found that mice deficient in macrophage elastase (MMP-12) did not develop CS-induced emphysema, highlighting the importance of this metalloprotease and suggesting a possible therapeutic approach. MMP-12 is mainly produced by macrophages and has been correlated with the destruction and remodeling of extracellular matrix components in humans and animal models of emphysema (Shipley et al., 1996).

We have already described that the administration of rBmTI-A, a serine protease inhibitor from the cattle tick *Rhipicephalus* (*B.*) *microplus*, can prevent and attenuate elastase-induced emphysema in mice, and we related this finding to the fact that the rBmTI-A inhibitor was able to interfere with the mechanism of metalloprotease 12 (MMP-12) activation through inhibition of neutrophil elastase, in this emphysema model (Lourenço et al., 2014). Also, it was described that rBmTI-A is capable of inhibiting trypsin, human neutrophil elastase, human plasma kalikrein and human plasmin (Soares et al., 2016).

Therefore, given that tobacco smoking is one of the main risk factors for COPD development (GOLD, 2015), we decided to verify the effects of this serine protease inhibitor in mice exposed to CS.

**Materials and methods**

This study was approved by the Review Board for human and animal studies from School of Medicine of University of São Paulo (São Paulo, Brazil). Six- to-eight week old male C57BL/6 mice (20-25 g) were provided by the central animal facility of this institution. All of the animals in the study received human care in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication, Eighth Edition, 2011) (Project Number 119/13).

**Induction of emphysema**

To induce pulmonary emphysema, CS exposure was performed in an inhalation chamber (28 L) previously described by Biselli et al. (2011) with two inlets for synthetic air and smoke supplies. An airflow of 2 L/min was maintained inside the chamber and, in a second inlet, the synthetic airflow passed through a Venturi System connected to a lit cigarette, which suctioned the cigarette smoke to inside the chamber. This flow rate produced CO levels ranging from 250 to 350 ppm. Animals were exposed to commercially filtered cigarettes (0.8 mg of nicotine, 10 mg of tar and 10 mg of CO per cigarette), with total particulate matter concentration of 354.8±50.3 μg/m³/day (Toledo et al., 2012) and carboxyhemoglobin concentration in mice exposed to CS was kept at 10% (±1.3%). Mice allocated to the CS exposure groups were kept in the chamber maintaining these CO levels twice a day, 30 min each exposition, 5 days per week over 12 weeks. Animals were exposed to 12 (±1) cigarettes at each one of the 30 min exposure. Control groups were exposed to room air.

**Preparation and administration of rBmTI-A inhibitor**

The rBmTI-A is described as a serine protease inhibitor from the cattle tick *Rhipicephalus* (*B.*) *microplus*, an important bovine ectoparasite from tropical and subtropical regions of the world, especially common in Brazil (Willadsen and Jongejan, 1999). Animals received a nasal instillation of 35.54 pmol of the recombinant inhibitor (rBmTI-A) in 50 μl of Saline Solution 0.9%, as we previously described (Loureno et al., 2014), following the protocol shown in Fig. 1. The control animals were treated with 50 μl of saline solution (0.9%).

**Study design**

To test the rBmTI-A therapeutic effect, animals were divided into four groups, according to the exposure and administration protocol. C57BL/6 mice were exposed to CS and the control groups were exposed to room air. These animals were treated with the rBmTI-A protease inhibitor (Control-rBmTI-A n=7 and CS-rBmTI-A groups n=8) or its vehicle, saline solution (0.9%) (Control-VE n=8 and CS-VE groups n=6).

**Respiratory mechanics**

Twenty-four hours after the last exposure, animals were deeply anesthetized by an intraperitoneal injection of thiopental (70 mg/kg), tracheostomized and then connected to a ventilator for small animals (Flexivent, Scireq, Montreal), with a tidal volume of 10 mL/kg and breathing frequency of 120 breaths/min. The animals were paralyzed using pancuronium bromide (0.2 mg/kg) to perform the respiratory mechanics protocol. The resistance of airways (Raw), tissue damping (Gtis) and tissue elastance (Htis) parameters were evaluated using a forced oscillation technique, based on the previously described model (Hantos et al., 1992).

**Lung biopsies preparation**

Animals were euthanized by exsanguination of the abdominal aorta and the thoracic cavity was opened to remove the lungs, which were infused with formalin through the trachea at a constant pressure of 20 cmH₂O for 24 h (Lopes et al., 2013; Robertoni et al., 2015).

Lung tissue sections of 5 μm were stained with H&E for evaluation of mean linear intercept (Lm) and density of polymorphonuclear cells (PMN), and also stained with...
Sirius Red and Resorcin-Fuchsin to evaluate the volume proportion of collagen and elastic fibers respectively, in lung parenchyma (Dolhnikoff et al., 1999).

**Immunohistochemistry**

The tissue sections were deparaffinized and hydrated. After blocking the endogenous peroxidase activity, an antigen retrieval step was performed with high-temperature citrate buffer (pH=6.0). All primary antibodies used in this study, except the anti MAC-2 (Cedarlene Laboratories, Canada), were from Santa Cruz Biotechnology, USA. The following dilutions were used: rat monoclonal IgG anti MAC-2 (Clone M3/38 1:5000); goat polyclonal IgG anti MMP-12 (SC-8839, 1:500); goat polyclonal IgG anti MMP-9 (SC-6840, 1:200); rabbit polyclonal IgG anti TIMP-1 (SC-5538, 1:100); goat polyclonal IgG anti gp91phox (SC-5827, 1:400). The secondary antibodies were the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA), according to the respective primary antibody. A 3,3’-diaminobenzidine (DAB, Sigma Chemical Co., St. Louis, MO, USA) was used as a chromogen and the sections were counterstained with Harris’s hematoxylin.

**Morphometry**

For conventional morphometry, an eyepiece with a coherent system of 50 lines, 100 points and a known area was attached to an ocular microscope (Weibel et al., 1966). All lung histological samples were analyzed in a blinded fashion and 15 non-overlapping fields were assessed per histological sample for each measurement. The mean linear intercept (Lm) was performed at 200× magnification and it was counted the number of times that lines of the eyepiece intercepted the alveolar walls. The Lm values were expressed in microns (μm) and were calculated by the relation between the sum of all segments of the eyepiece, and the average number of times that the lines intersected the alveolar walls, as we previously described (Lourenço et al., 2014).

To evaluate the volume proportion of collagen and elastic fibers, the number of points hitting a specific fiber in the alveolar parenchyma were counted and compared with the number of points hitting the alveolar tissue in each field, to generate each proportion at 400× magnification. At the same magnification, the number of macrophages and positive cells for MMP-12, MMP-9, TIMP-1 and anti-glycosylated 91-kDa glycoprotein (gp91phox) in the alveolar parenchyma were also assessed by a point-counting technique. For PMN cell quantification, a 1,000× magnification was used and the number of cells in each field was counted and divided by the number of points hitting the lung parenchyma. The results were expressed as cells per square micrometers.

**Statistical analysis**

All data are expressed as the means and ± standard error (SE). Statistical analysis was performed using SigmaStat software (SPSS Inc. Chicago, Illinois, USA). The values were compared using a One-Way ANOVA followed by all pairwise multiple comparison procedures (Tukey test). A p-value of less than 0.05 was considered to be significant.

**Results**

**Respiratory mechanics**

There was a decrease in Htis values only in CS-VE group compared to the other groups (*p≤0.013) (Fig. 2A), although there were no differences between experimental groups for the Gtis and Raw parameters (Fig. 2B,C).

**Lm evaluation**

The mean linear intercept values were increased in CS-VE group compared to the other groups (*p<0.013), suggestive of emphysema in the CS non treated group. Administration of the rBmTI-A inhibitor decreased Lm values in CS-rBmTIA group to Control levels (Fig. 3A).

**Collagen and elastic fibers remodeling**

The same pattern can be observed in Fig. 3B for percentage of collagen fibers in parenchyma. The CS-VE group have higher amounts of collagen fibers when compared to the other groups (p<0.002). There was an increase in elastic fibers in CS-VE group compared to the other groups (p<0.023) and the rBmTI-A administration only attenuated these values in the CS-rBmTIA (CS-rBmTIA x Control-rBmTIA p=0.002).
**Immunohistochemistry and PMN cells**

The quantification of macrophages (Fig. 4A) in parenchyma showed an increase of positive cells for these cell type in both groups exposed to CS compared to the control groups (p<0.017). There was an increase in density of MMP-12 (Fig. 4C) in CS-VE group compared to the others (p<0.001) and administration of rBmTIA decreased the number of these cells (p<0.017 compared to Control groups). Although there was no difference between experimental groups for density of polymorphonuclear cells in parenchyma (Fig. 4B), the analysis of MMP-9 positive cells in parenchyma (Fig. 4D) showed higher amounts in both groups exposed to CS (p<0.001 compared to Control groups; p=0.013 compared to Control-VE group). Furthermore, there was an increase in TIMP-1 positive cells (Fig. 4E) in CS-rBmTIA compared to the VE groups (p<0.013) and an increase in Control-rBmTIA group compared to Control-VE (p<0.001). The gp91phox analyses (Fig. 4F) showed an increase of this oxidant component only in CS-rBmTIA group compared to the other groups (p<0.008). Photomicrographs of specific immuno-stainings for MMP-12, TIMP-1 and gp91phox, are demonstrated in Fig. 5.

**Discussion**

We verified that the rBmTIA-A inhibitor administration during the emphysema-induced protocol inhibited the emphysema development in mice with a concomitant improvement in respiratory function and a decrease in the parenchymal fibers remodeling.

Although the majority of studies have demonstrated the presence of emphysema with 6 months of CS exposure (Wright and Churg, 1990; Haumataki et al., 1997; Shapiro et al., 2003; Churg et al., 2007, 2009), in this present study we verified an alveolar enlargement...
Fig. 3. Measurement of mean linear intercept and volume proportion of collagen and elastic fibers in parenchyma. A. There was an increase in Lm values only in CS-VE groups, and the rBmTI-A reduced this response to control levels (*p<0.013 compared to the other groups). The same pattern can be observed in (B) collagen fibers (*p<0.002 compared to the other groups). C. There was an increase in elastic fibers in CS-VE group, and the administration of rBmTI-A attenuated this response (*p<0.023 compared to the other groups; **p=0.002 compared to Control-rBmTIA). For all measurements: Control-VE n=8; Control-rBmTIA n=7; CS-VE n=6; CS-rBmTIA n=8. Values are means ± SE. Pictures illustrate the results found for the evaluation of Lm (scale bar: 100 µm) and volume proportion of collagen and elastic fibers (scale bar: 50 µm).
Fig. 4. Density of positive cells in pulmonary parenchyma for macrophages (A), MMP-12 (C), MMP-9 (D), TIMP-1 (E), gp91phox (F) and the number of polymorphonuclear cells (B). A. * p<0.017 compared to the control groups (Control-VE n=8; Control-rBmTIA n=6; CS-VE n=6; CS-rBmTIA n=8). B. No statistical difference between experimental groups (Control-VE n=8; Control-rBmTIA n=5; CS-VE n=8; CS-rBmTIA n=6). C. *p<0.001 compared to the other groups; **p<0.017 compared to Control groups (Control-VE n=8; Control-rBmTIA n=6; CS-VE n=6; CS-rBmTIA n=7). D. *p<0.001 compared to Control groups; **p=0.013 compared to Control-VE group (Control-VE n=7; Control-rBmTIA n=6; CS-VE n=6; CS-rBmTIA n=7). E. *p=0.013 compared to VE groups; **p<0.001 compared to Control-VE group (Control-VE n=8; Control-rBmTIA n=6; CS-VE n=6; CS-rBmTIA n=8). F. *p<0.008 compared to the other groups (Control-VE n=8; Control-rBmTIA n=6; CS-VE n=6; CS-rBmTIA n=8). Values are means ± SE.
after 3 months (2 times a day). It is important to note that most studies that expose animals to CS for 6 months used a reduced number of cigarettes (from 2 to 7) in only one exposure per day. Even so, Wright and Churg (1990) exposed guinea pigs to 10 cigarettes per day for 1, 3, 6 and 12 months, and demonstrated an increase in Lm

![Immunohistochemistry photomicrographs.](image)

**A) MMP-12**

**B) TIMP-1**

**C) GP91phox**

Fig. 5, Immunohistochemistry photomicrographs. A representative area is shown from the pulmonary parenchyma of the experimental groups for MMP-12 (A), TIMP-1 (B) and gp91phox (C) immunohistochemistry reactions. Scale bar: 50 µm.
since the third month of CS exposure. Also, Hautamaki et al. (1997) found airspace enlargement in mice after 3 to 4 months of exposure to 2 nonfiltered cigarettes per day.

The importance of macrophages and MMP-12 in the development of both CS and elastase induced emphysema have been extensively described (Hautamaki et al., 1997; Shapiro, 1998; Belvisi and Bottomley, 2003; Churg et al., 2012). In our study, we also verified an increase in the density of macrophages and MMP-12 positive cells in both of the CS exposed groups. Although the rBmTI-A administration did not reduce the macrophage density, it was responsible for a decrease in the density of MMP-12 positive cells in animals that received rBmTI-A administration.

There were no differences among the groups for the number of polymorphonuclear cells, suggesting that there is no neutrophilic infiltration within the parenchyma in this model. However, many studies showed the increase in neutrophils only in early events during the emphysema development preceding the increase in Lm and the worsening in respiratory function (Dhami et al., 2000; Churg et al., 2004). Ofu1ue et al. (1998) described an increase in interstitial neutrophils in rats only at the first month of CS exposure, but it was reduced to control levels at second month. It is possible that in this present study we did not detect differences among the groups for neutrophil amounts because we performed the analysis only after 3 months of exposure.

Although the analysis of positive cells for MMP-9, a metalloprotease produced by neutrophils, eosinophils, alveolar macrophages, mast and epithelial cells (Mahadeva and Shapiro, 2002) showed an increase for both groups exposed to CS, the rBmTI-A administration did not interfere in these results. Moreover, the density of MMP-12 positive cells was higher compared to MMP-9 even when animals received the rBmTI-A administration, reinforcing the importance of MMP-12 for emphysema development in this CS-induced model.

The TIMPs are a family of endogenous inhibitors that regulate the equilibrium between proteolysis and proteolysis inhibition, providing the integrity of the extracellular matrix components (Belvisi and Bottomley, 2003; Gueders et al., 2006). Deregulation between TIMPs and MMPs leads to an exaggerated extracellular matrix turnover, typical of remodeling processes (Gueders et al., 2006). In this study, we observed that the rBmTI-A administration induced in both groups an increase in TIMP-1 positive cells. However, the VE groups showed reduced values compared to the rBmTI-A groups, suggesting the importance of this inhibitor administration to this elevated TIMP production.

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**Fig. 6.** Diagram summarizing the MMP-12 related mechanisms involved in CS-induced emphysema observed in our study.
Shapiro et al. (2003) have previously described several interactions between neutrophil and macrophage elastases, since each enzyme inactivated the endogenous inhibitor of the other. These authors reported a decrease in macrophage elastase activity and macrophage accumulation in neutrophil elastase deficient mice, which was related to the inability of the system to degrade TIMP-1.

Such results are in agreement with ours, once we have observed that the administration of rBmTI-A has interfered with the density of TIMP-1 positive cells. It is possible that the rBmTI-A can act early inhibiting the neutrophilic elastase since the first month of CS-exposure, preventing TIMP-1 degradation.

Although TIMP-1 is described as the main inhibitor of MMP-2 and -9 in humans (Finlay et al., 1997; Lim et al., 2000; Russell et al., 2002; Gueders et al., 2006), Shapiro et al. (2003) have already demonstrated in animal models, that TIMP-1 is the main TIMP released in lungs in response to cigarette smoke exposure, and that the MMP-12 is the main metalloprotease involved in this CS-induced model of emphysema (Churg et al., 2007, 2012), both produced and released in majority by alveolar macrophages. These facts reinforce the relation seen in our study, where the decrease in MMP-12 positive cells observed in animals exposed to CS and that received the rBmTI-A administration could also be associated to this tissue inhibitor of metalloprotease.

NADPH oxidase is another enzyme implicated in the regulation of MMP-12 through the generation of reactive oxygen species. Kassin et al. (2005) showed that the gp91phox (a subunit of the NADPH oxidase)-deficient cells had greater MMP-12 activity than wild type macrophages, leading them to propose that oxidizing intermediates from NADPH, downregulating the activity of MMP-12 by modifying specific amino acids. Thus, the loss of this downregulation leads to matrix degradation and emphysema, as well as higher amounts of gp91phox that can control and maintain the MMP-12 activity. We also evaluated the effects of rBmTI-A administration upon the density of gp91phox positive cells, an oxidant component. We found that rBmTI-A administration only in CS exposed animals produced increases in the density of gp91phox positive cells. Further experiments are now needed to clarify the role of rBmTI-A in this oxidant component increase.

Thus, in agreement with Kassin et al. (2005), it is possible that the reduction of MMP-12 positive cells in animals exposed to CS and that received rBmTI-A administration compared to CS-VE could also be attributed to an increase in gp91phox in parenchyma. The MMP-12 related mechanisms observed in this study are summarized in the diagram below (Fig. 6).

By comparing the effects of rBmTI-A administration between the CS and elastase induced models (Lourenço et al., 2014), it is important to consider the differences between these animal models. We have already demonstrated the different patterns of parenchymal remodeling (Lopes et al., 2013), which reflect the significant differences in pathophysiological mechanisms mediating the emphysema development between both models. Although the elastase-induced model could induce a greater parenchymal fibers injury and remodeling compared with the CS model, the CS-induced model induces a larger persistent inflammatory process (Wright et al., 2008). However, despite the differences in both models, the attenuation of emphysema could be attributed to a decrease in MMP-12 positive cells density.

In summary, our findings for the rBmTI-A administration in a CS-induced emphysema model reinforces the importance of MMP-12 in the development of this disease, suggesting this MMP as a potential target for future therapeutic approaches for emphysema.

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Conflict of interest: Authors declare no potential conflicts of interest

Ethical Approval: All procedures performed in this study involving animals were in accordance with the ethical standards of the institution or practice at which this study was conducted.

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