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Original Contribution

Oxidative stress and nitrosative stress are involved in different stages of proteolytic pulmonary emphysema

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ABSTRACT

Our aim was to investigate the role of oxidative stress in elastase-induced pulmonary emphysema. C57BL/6 mice were subjected to pancreatic porcine elastase (PPE) instillation (0.05 or 0.5 U per mouse, i.t.) to induce pulmonary emphysema. Lungs were collected on days 7, 14, and 21 after PPE instillation. The control group was sham injected. Also, mice treated with 1% aminoguanidine (AMG) and inducible NO synthase (iNOS) knockout mice received 0.5 U PPE (i.t.), and lungs were analyzed 21 days after. We performed bronchoalveolar lavage, biochemical analyses of oxidative stress, and lung stereology and morphometry assays. Emphysema was observed histologically at 21 days after 0.5 U PPE treatment; tissues from these mice exhibited increased alveolar linear intercept and air-space volume density in comparison with the control group. TNF-α was elevated at 7 and 14 days after 0.5 U PPE treatment, concomitant with a reduction in the IL-10 levels at the same time points. Myeloperoxidase was elevated in all groups treated with 0.5 U PPE. Oxidative stress was observed during early stages of emphysema, with increased nitrite levels and malondialdehyde and superoxide dismutase activity at 7 days after 0.5 U PPE treatment. Glutathione peroxidase activity was increased in all groups treated with 0.5 U PPE. The emphysema was attenuated when iNOS was inhibited using 1% AMG and in iNOS knockout mice. Furthermore, proteolytic stimulation by PPE enhanced the expression of nitrotyrosine and iNOS, whereas the PPE+AMG group showed low expression of iNOS and nitrotyrosine. PPE stimulus also induced endothelial (e) NOS expression, whereas AMG reduced eNOS. Our results suggest that the oxidative and nitrosative stress pathways are triggered by nitric oxide production via iNOS expression in pulmonary emphysema.

Chronic obstructive pulmonary disease (COPD) is characterized by chronic bronchitis and pulmonary emphysema. Emphysema is the major cause of chronic morbidity and mortality in COPD patients worldwide [1]. Emphysema is characterized by air-space enlargement, accompanied by the destruction of parenchymal structure. The breakdown of the extracellular matrix in the parenchymal tissues occurs because of the inflammatory response, in which inflammatory cells are recruited to the lungs. There, the inflammatory cells release a large quantity of proteinases, exceeding the proteinase inhibitor defenses of the lung; this phenomenon is the basis of the well-described proteinase–antiproteinase hypothesis [2]. Beyond proteinase release, neutrophils and activated macrophages also release reactive oxygen species owing to the respiratory burst that occurs during the inflammatory process. These cells also secrete cytokines, potentiating further inflammation. It is known that both proteolytic stimulus and oxidative stress are involved in the pathogenesis of pulmonary emphysema.

Cigarette smoking, the main risk factor for the development of emphysema, has long been considered responsible for the oxidative stress involved in this process. However, recent studies have demonstrated that endogenous oxidative stress is involved in proteolytic emphysema in several rodent models. Most of these studies have investigated genes that are related to the cellular antioxidant response, such as NF-E2-related factor 2 [3],

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thioredoxin 1 [4], and SOD3 (extracellular superoxide dismutase, or ECSOD) [5]. In contrast, our study focused on evaluating the antioxidant response in the lungs after porcine pancreatic elastase stimulation at various time points of emphysema establishment. We also investigated the participation of the nitrosative stress pathway as an aggravator of protease-induced pulmonary emphysema. A very recent study has demonstrated that inducible nitric oxide synthase (iNOS) inhibition reverses the pulmonary emphysema induced by cigarette smoke [6]. However, we hypothesized that peroxynitrite synthesis, from the nitric oxide and superoxide anion reaction, contributes to the development of pulmonary emphysema, disturbing the antioxidant defenses and generating oxidative damage at the cellular level.

Material and methods

All procedures were approved by the Institutional Ethics Committee of the Federal University of Rio de Janeiro (DAHEICB 063).

Study I

Five groups of C57BL/6 mice ($n=6$) were used. Mice were anesthetized and subjected to an intratracheal instillation of either 0.05 or 0.5 U of porcine pancreatic elastase (PPE; Sigma–Aldrich) or saline (control group). Animals were sacrificed at 7, 14, and 21 days after treatment to better comprehend the moment of the appearance of the emphysematous lesions and at which moment the oxidative stress is crucial for the emphysema development.

Study II

To assay the role of nitric oxide in the pathogenesis of emphysema, we inhibited iNOS by treating the animals with 1% aminoguanidine (AMG), provided in the drinking water. AMG treatment began 1 day before emphysema induction and continued throughout the entire emphysema time course (to 21 days after 0.5 U of PPE). Mice were randomized into four groups ($n=5$): control, AMG, PPE, and PPE+AMG.

Study III

To study the role of inducible nitric oxide synthase in the pathogenesis of emphysema, C57BL/6 wild-type and iNOS knockout mice were subjected to an intratracheal instillation of 0.5 U of PPE and sacrificed 21 days after the treatment. Mice were randomized into four groups ($n=5$): control, PPE, control iNOS KO, and PPE iNOS KO.

Lung histology and quantification of emphysema

Mice were sacrificed by cervical displacement. Left lungs were removed and fixed with 4% paraformaldehyde. The tissue was embedded in paraffin, and 5-μm-thick sections were stained with hematoxylin and eosin (H&E). Air-space enlargement was quantified by the mean linear intercept length of the distal air spaces (L_m) in 30 randomly chosen fields of tissue sections per group [7]. The surface density of alveolar septa (S_v[alvsept]), volume density of alveolar septa (V_v[alvsept]), and air-space volume density (V_v[air space]) were calculated by counting the number of structures that were intersected by the test system (partial points, P_p) in 30 randomly chosen fields per group [8].

Measurements in bronchoalveolar lavage (BAL)

BAL was performed immediately after sacrifice (3×0.5 ml saline). The trachea was exposed and cannulated to allow access to the airway. The BAL fluid was kept on ice. Aliquots were reserved for cell counts (performed under a microscope), and the remaining BAL was centrifuged (600*g*). Aliquots of the supernatant were subjected to ELISA to measure secreted TNF-α and IL-10, Bradford assays to measure protein content [9], myeloperoxidase (MPO) assay [10], and a colorimetric hydroxyproline assay (OHpro) [11,12]. An outline of the MPO and OHpro assays can be seen in Table 1. Reactive oxygen species (ROS) detection was performed in BAL cells following the adapted nitroblue tetrazolium chloride (NBT) protocol [13]. Briefly, ROS produced by cells reacted with NBT, generating blue formazan. Optical density was read in a microplate reader (Bio-Rad Model 550; Hercules, CA, USA) at 630 nm. All assays were conducted as previously described.

Measurements in lung homogenates

Right lungs were removed and homogenized in potassium phosphate buffer. The homogenate was centrifuged (600*g*) and the supernatant was removed. Protein concentration was estimated using the Bradford method [9]. The outline of the biochemical analyses can be seen in Table 1. Nitrite levels were determined by a method based on the Griess reaction [14]. SOD activity was determined as described by Bannister and Calabrese [15]. Glutathione peroxidase (GPx) activity was measured as described by Flohe and Gunzler [16]. Lipid peroxidation was measured using a thiobarbituric acid-reactive substances assay (TBARS) as a marker of oxidative damage [17]. Western blots were used to analyze the expression of iNOS, endothelial (e) NOS, and nitrotyrosine. The antibodies against iNOS (Cayman Chemical Co., Ann Arbor, MI, USA), eNOS, and nitrotyrosine (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1/1000. Tubulin (Santa Cruz Biotechnology) was used as an internal control. Equal loading of the samples was ensured by protein quantification

Table 1
Outline of biochemical analyses.

Analysis	Scope	7 days	14 days	21 days HD	21 days LD
MPO	Lung injury related to neutrophils	↑↑↑	↑	↑↑	—
OHpro	Collagen breakdown	↑↑	—	↑↑↑	—
Nitrite	Nitric oxide	↑	—	↑↑	—
SOD	Antioxidant defense, mainly against superoxide anion	↑	—	—	—
GPx	Antioxidant defense, mainly against hydroperoxides	↑↑↑	↑↑↑	↑↑↑	↑↑↑
TBARS	Lipid peroxidation	↑	↑	—	—

MPO, myeloperoxidase; OHpro, hydroxyproline; SOD, superoxide dismutase; GPx, glutathione peroxidase; TBARS, thiobarbituric acid-reactive substances. 21 days HD, high dose of PPE, like 7 and 14 days; 21 days LD, low dose of PPE. All comparisons are relative to the control group.

before SDS–PAGE; membranes were also reprobed for tubulin to confirm equal loading.

Statistical analyses

The data were compared using one-way ANOVA followed by Bonferroni post hoc test (means ± SEM, $p < 0.05$). Statistical analyses were performed in GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Results

Lung emphysema induced by PPE is time- and dose-dependent

To evaluate the various stages of emphysema development and determine whether it is PPE dose-dependent, we exposed mice to 0.5 and 0.05 U of PPE and observed the lung histology at 7, 14, and 21 days after instillation. For the lowest dose (0.05 U of PPE), we analyzed only the 21-day time point. H&E-stained histological sections revealed normal lung structure in the control group. At 7 days post-PPE instillation (0.5 U), no changes in the lung parenchyma were observed. At 14 days post-PPE, some alveolar increase was observed, and at 21 days, complete emphysema had occurred, with the significant enlargement of alveolar spaces distributed throughout the parenchyma (Fig. 1A–E). To further characterize the establishment of emphysema, histological findings were quantified in the lung slices. As shown in Table 2, all histological abnormalities typical of emphysema were observed in the tissues collected at 21 days post-PPE 0.5 U instillation, including increased L_m and Vv[air space], accompanied by decreased Sv[alvsept] and Vv[alvsept].

PPE (0.5 U) triggers chronic inflammatory response with early cytokine modulation in BAL

Cell counts were conducted in BAL to evaluate leukocyte migration; increased numbers of cells were found only at 21 days post-PPE 0.5 U instillation (Fig. 2A). However, the response at the cytokine secretion level occurred at earlier time points. The proinflammatory TNF-α was elevated at 7 and 14 days post-PPE 0.5 U instillation, whereas the anti-inflammatory IL-10 was reduced at the same time points (Fig. 2B and C). An increase in myeloperoxidase was observed at 7 days ($p < 0.001$) and persisted at 14 and 21 days after the instillation of 0.5 U of PPE (Fig. 2D, Table 1), characterizing a high number of activated neutrophils in the airways. In contrast, the hydroxyproline levels detected in the BAL were increased at 7 days ($p < 0.01$), reduced to baseline (i.e., levels similar to those of the control group) at 14 days, and increased again ($p < 0.001$) at 21 days after instillation of 0.5 U of PPE (Fig. 2E, Table 1), showing a cyclic extracellular matrix remodeling by turnover of collagen. None of the evaluated parameters were altered in the group treated with 0.05 U of PPE for 21 days.

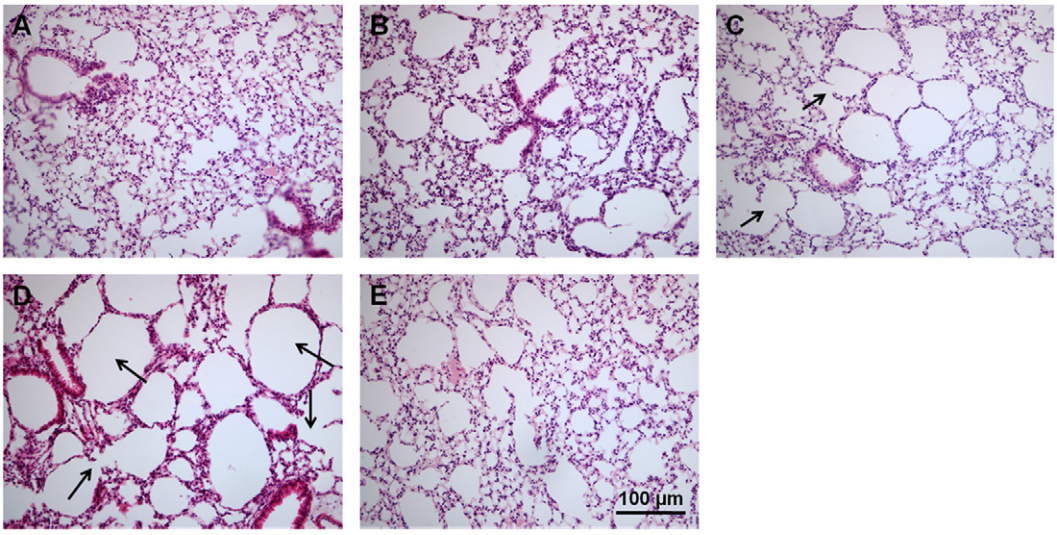

Fig. 1. H&E-stained lung parenchyma. The tissues shown are from (A) mice in the saline control group, at 21 days; (B–D) mice treated with 0.5 U of PPE, at 7, 14, and 21 days postinstillation, respectively; and (E) mice treated with 0.05 U of PPE, at 21 days postinstillation. Arrows indicate septal wall tissue destruction and consequent alveolar enlargement. Scale bar, 100 µm.

Table 2
Lung morphological and stereological quantifications after PPE-induced emphysema.

	Control	PPE 0.5 U			PPE 0.05 U, 21 days
		7 days	14 days	21 days	
L_m (µm)	48.12 ± 1.25	52.29 ± 3.86	67.19 ± 5.77	89.16 ± 6.75*	51.97 ± 1.30
Sv (µm^{-1})	0.083 ± 0.002	0.078 ± 0.005	0.062 ± 0.006	0.046 ± 0.004*	0.077 ± 0.002
Vv[alvsept] (%)	53.7 ± 0.86	51.6 ± 2.57	45.5 ± 1.83	32.9 ± 3.19*	47.5 ± 2.06
Vv[air space] (%)	46.2 ± 0.86	48.4 ± 2.57	54.5 ± 1.83	67.1 ± 3.19*	52.5 ± 2.06

L_m, mean linear intercept length of distal air spaces; Sv, surface density of alveolar septa; Vv[alvsept], volume density of alveolar septa; Vv[air space], volume density of air spaces; PPE, porcine pancreatic elastase. The results shown represent the means ± SEM; n=6/group.

* $p < 0.01$ compared with control group.

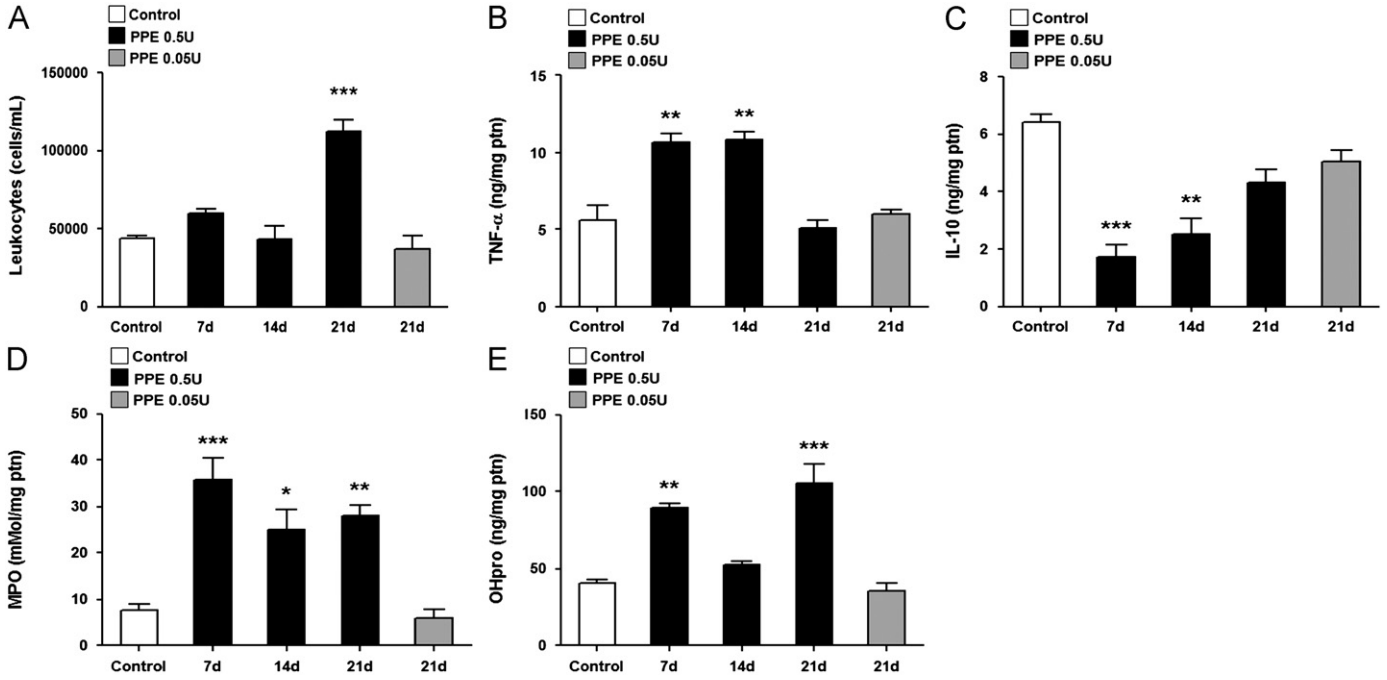

Fig. 2. Inflammatory parameters involved in the establishment of emphysema. BAL samples were used to detect (A) total leukocytes, (B) TNF-α cytokine levels, (C) IL-10 cytokine levels, (D) myeloperoxidase activity, and (E) hydroxyproline levels. Significant differences compared to the control: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. $n=6$/group.

Proteolytic stimulation by PPE triggers oxidative stress in early lung injury

Nitrite levels can be used as an indirect measurement of nitric oxide, a physiologic molecule that can be enhanced in the inflammatory process and contribute to oxidative and nitrosative stress. Nitrite levels were increased at 7 days after the instillation of 0.5 U of PPE ($p<0.05$), returned to baseline at 14 days, and increased again ($p<0.01$) at 21 days (Fig. 3A, Table 1). Meanwhile, antioxidant enzymes exhibited different temporal response patterns. Superoxide dismutase activity was increased only at 7 days after instillation of 0.5 U of PPE ($p<0.05$; Fig. 3B, Table 1), whereas glutathione peroxidase was enhanced at all time points tested ($p<0.001$; Fig. 3C, Table 1). Further demonstrating that oxidative stress is more evident at early time points, the groups at 7 and 14 days after instillation of 0.5 U of PPE exhibited elevated levels of TBARS ($p<0.05$; Fig. 3D), a marker of oxidative damage.

Aminoguanidine treatment attenuates lung emphysema

To verify that nitric oxide is involved in the elastolytic model of emphysema, mice subjected to the instillation of 0.5 U of PPE were treated with 1% AMG for 22 days, beginning 1 day before the PPE treatment. Lung histology showed that mice treated with 1% AMG alone were indistinguishable from those in the control group (Fig. 4A), with normal alveolar spaces and preserved septa (Fig. 4B). PPE-treated mice exhibit lesions typical of emphysema, with increased alveolar spaces and evident septal destruction (Fig. 4C), as described above. However, the PPE+AMG-treated mice showed relatively well-preserved lung parenchyma, with better maintained alveolar septa and consequently better preserved alveolar spaces than the PPE group (Fig. 4D). As shown in Table 3, all histological alterations typical of emphysema were observed at 21 days post-PPE 0.5 U instillation, including increased L_m and Vv[airspaces], accompanied by decreased Sv[alvsept] and Vv[alvsept]. However, treatment with 1% aminoguanidine effectively attenuated the effects of PPE on the lungs, and the PPE+AMG group was indistinguishable from the control group.

AMG decreases inflammatory infiltrate and protein nitration induced by PPE

BAL cell counts showed that PPE induced an increase in leukocyte migration ($p<0.001$) compared to the control group. In contrast, the cell counts in the PPE+AMG group were similar to those in the controls and were reduced ($p<0.001$) compared to the PPE group (Fig. 5). Western blotting analysis showed that treatment with 1% aminoguanidine partially inhibited iNOS expression (Fig. 6). Nevertheless, AMG effectively prevented PPE-induced iNOS overexpression in the PPE+AMG group. Surprisingly, the PPE+AMG group also exhibited a reduction in eNOS expression compared to the PPE group. As a consequence of iNOS and eNOS overexpression, the PPE group exhibited elevated nitrotyrosine, a marker of nitrosative stress, whereas AMG treatment effectively prevented protein nitration; lower nitrotyrosine expression was observed in the PPE+AMG group compared to the PPE group.

iNOS deficiency protects against PPE-induced emphysema

To better characterize the role of iNOS in the progression of emphysema a separate group of C57BL/6 wild-type and iNOS$^{-/-}$ mice were exposed to PPE. Lung histology and morphometry/stereology were assayed to evaluate the pulmonary architecture. Total leukocyte numbers were quantified and ROS levels were analyzed as a parameter of oxidative stress. Lung histology showed that control iNOS$^{-/-}$ mice were similar to the control wild-type group (Fig. 7A), with normal alveolar spaces and preserved septa (Fig. 7B). PPE-treated mice exhibited lesions typical of emphysema, with increased alveolar spaces and evident septal destruction (Fig. 7C), as described above. However, PPE iNOS$^{-/-}$ mice showed intact alveolar septa and consequently better preserved alveolar spaces than the PPE group (Fig. 7D). Table 4 confirms all the typical changes in the PPE WT group, with

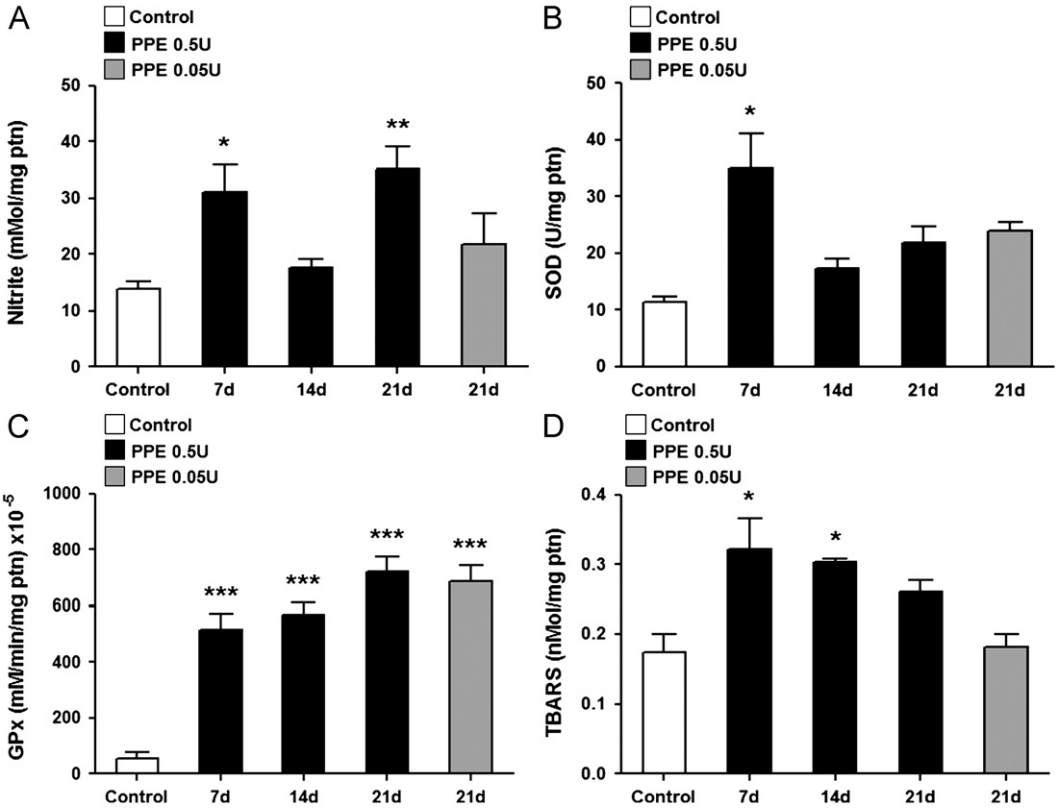

Fig. 3. Markers of oxidative stress involved in the establishment of emphysema. (A) Nitrite levels, (B and C) SOD and GPx activity, and (D) TBARS were measured in lung tissue samples. Significant differences compared to the control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. $n = 6$/group.

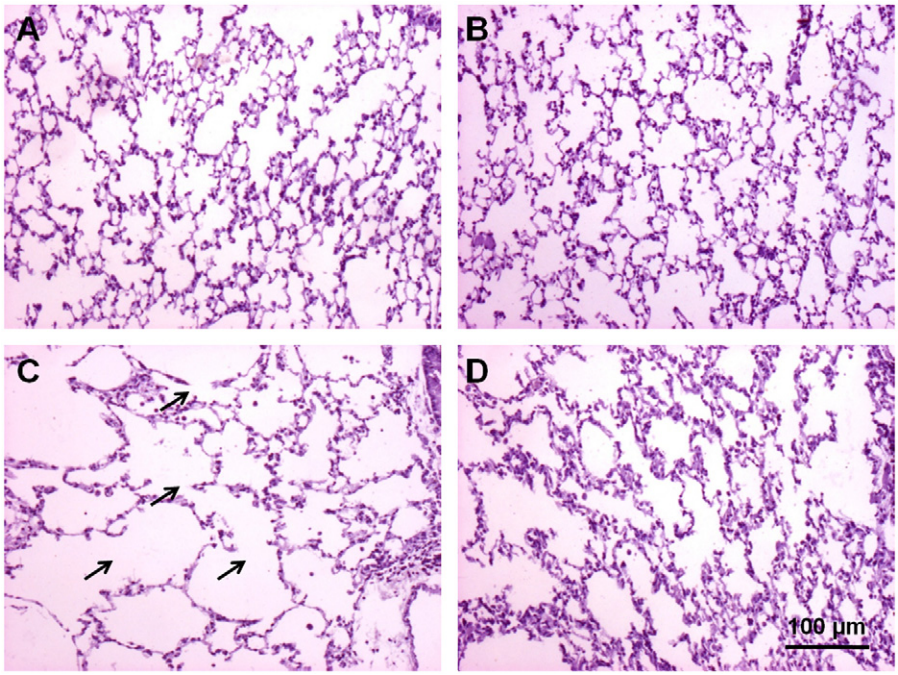

Fig. 4. H&E-stained lung parenchyma after 1% AMG treatment. The tissues shown are from (A) mice in the saline group, at 21 days postinstillation; (B) mice treated with 1% AMG in the drinking water; (C) mice treated with 0.5 U of PPE, at 21 days postinstillation; (D) mice treated with 0.5 U of PPE and treated with 1% AMG in the drinking water, at 21 days postinstillation. Arrows indicate septal wall tissue destruction and consequent alveolar enlargement. Scale bar, 100 µm.

a significant improvement in the PPE iNOS$^{-/-}$ group. The phenotype is better in PPE iNOS$^{-/-}$ mice than in the PPE WT group, because this group presented a reduction in total leukocyte numbers ($p < 0.01$) and ROS levels ($p < 0.001$; Fig. 7E and F).

Discussion

Our results demonstrate a critical interaction between different pathways involved in pulmonary emphysema, whereby proteolytic

Table 3
Lung morphological and stereological quantifications after PPE-induced emphysema and AMG treatment.

	Control	AMG	PPE	PPE+AMG
L_m (μm)	45.53 ± 1.79	49.96 ± 2.65	68.31 ± 1.55*	57.24 ± 6.47
Sv (μm^{-1})	0.088 ± 0.004	0.080 ± 0.004	0.059 ± 0.001*	0.065 ± 0.008
Vv[alvsept] (%)	49.20 ± 1.53	46.20 ± 1.16	39.67 ± 2.60*	42.67 ± 2.96
Vv[air space] (%)	50.80 ± 1.53	53.80 ± 1.16	62.00 ± 2.60*	57.33 ± 2.96

L_m, mean linear intercept length of distal air spaces; Sv, surface density of alveolar septa; Vv[alvsept], volume density of alveolar septa; Vv[air space], volume density of air spaces; PPE, porcine pancreatic elastase; AMG, aminoguanidine. The results represent the means ± SEM; $n=5$/group.

* $p < 0.05$ compared with control group.

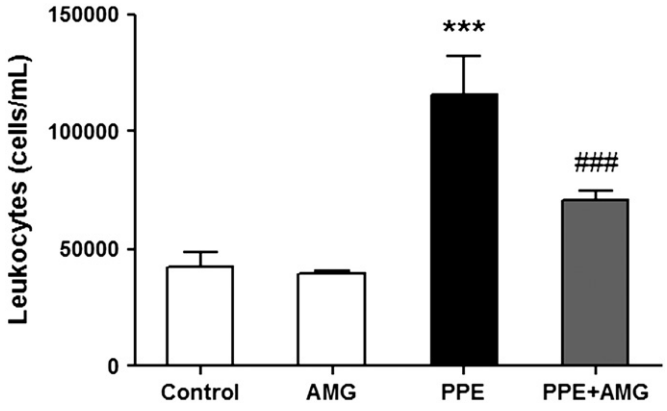

Fig. 5. The inhibition of iNOS by aminoguanidine treatment prevented inflammatory infiltration. Total leukocytes were counted. Significant difference compared to the control, ***$p < 0.001$, and significant difference compared to the PPE group, ###$p < 0.001$. $n=5$/group.

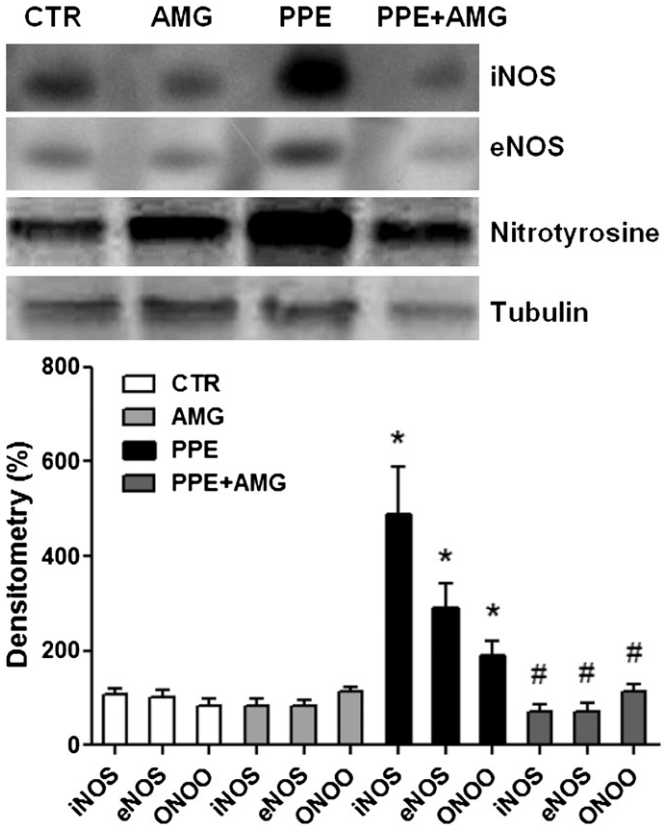

Fig. 6. The inhibition of iNOS by aminoguanidine treatment prevented nitrosative stress. Western blots were used to detect iNOS, eNOS, and nitrotyrosine. Each Western blot had its own tubulin loading control. Densitometry of the bands was calculated and normalized to loading control. Statistical difference compared to the control, *$p < 0.05$, and significant difference compared to the PPE group, #$p < 0.05$. $n=3$ or 4/group.

stimuli induced by elastase lead to oxidative stress and inflammation at different time points during the establishment of emphysema. An interesting fact is observed at 21 days after stimulation; at this time point the emphysema is completely installed and any changes linked to antioxidant enzyme activities are observed. The changes in antioxidant enzyme activities were observed at the initial stage of emphysema establishment, when the final lesions have yet to be developed. This demonstrates that oxidative stress participates in the evolution of pulmonary emphysema, even in a proteolytic model. We also demonstrate that intervention in nitric oxide synthesis may be a promising strategy for the treatment of emphysema because mice exposed to proteolytic stimuli suffered nitrosative stress and presented enhanced nitrotyrosine expression, a marker of nitrosative damage. Aminoguanidine treatment inhibited this process, thus preventing this complication and attenuating septal tissue destruction.

There is a rationale explaining the relationship between inflammation and oxidative stress in this model. When elastase is instilled and reaches the airways, tissue destruction processes are initiated. Then, inflammatory cells are activated and migrate to the alveolar spaces to neutralize the harmful stimulus [18]. However, the proteases and ROS are inherent by-products of activated leukocytes and contribute to the inflammatory response and parenchyma destruction [19–21]. Additionally, the direct increase in the oxidative burden produced by the release of oxygen radicals from inflammatory neutrophils and macrophages contributes to the establishment of an oxidative stress [22]. Even a single exposure to PPE is sufficient to trigger a continuous degradation process because peptides derived from the extracellular matrix (ECM) are chemotactic for inflammatory cells, suggesting that ECM destruction may be a self-perpetuating process [23].

The protocol used in this study effectively produced emphysematous lesions in a dose-dependent manner; a low dose of 0.05 U of PPE did not induce structural lung alterations. On the other hand, mice exposed to 0.5 U of PPE underwent typical emphysematous tissue destruction, as indicated by several morphological/stereological parameters. Increased L_m and decreased Sv and Vv[alvsept] demonstrate accelerated tissue destruction, increasing Vv[air space] to produce enlarged alveolar spaces. All of these histological measures are well-established features of pulmonary emphysema [24,25]. Associated with these histological changes, elevated leukocyte recruitment was observed in the BAL of the group observed 21 days after PPE instillation (0.5 U), i.e., the emphysema group, demonstrating that the presence of inflammatory cells is linked to emphysema [5]. However, leukocyte infiltration was not observed at 7 and 14 days after PPE instillation, even though an increase in TNF-α cytokine secretion

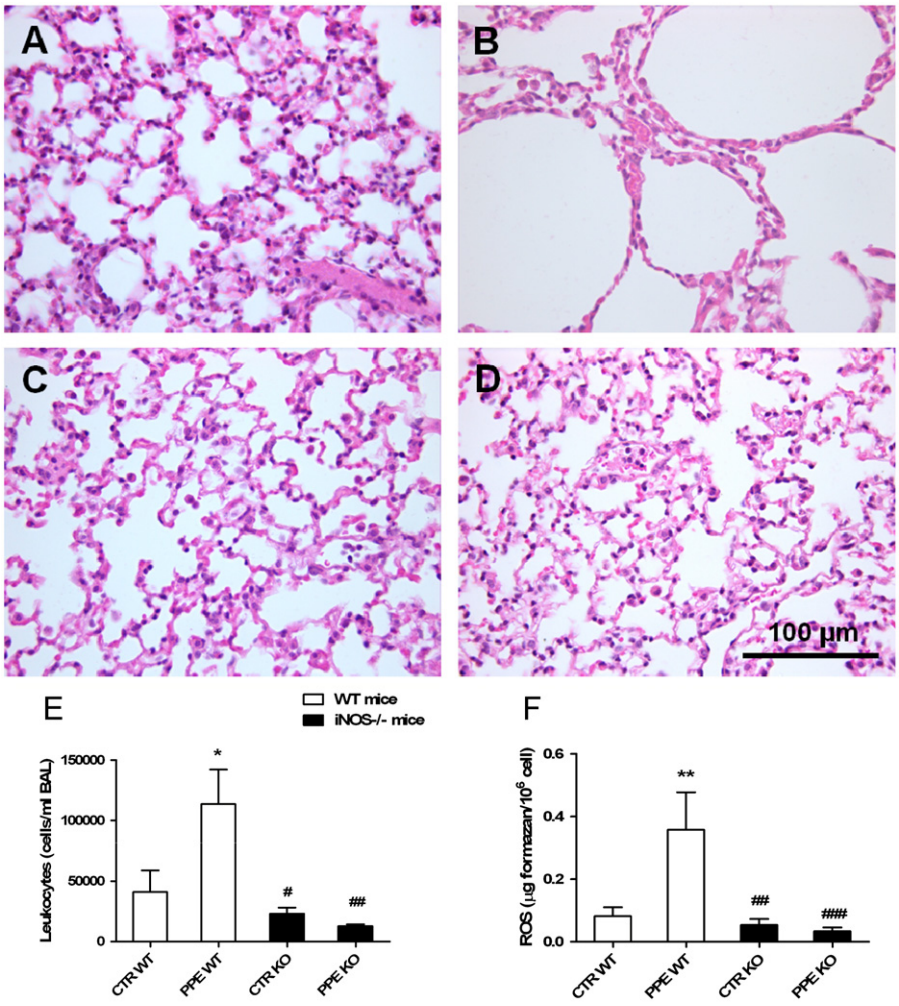

Fig. 7. iNOS-deficient mice are more protected against emphysema than wild type. Lung parenchyma H&E-stained sections 21 days postinstillation are shown. (A) Wild-type control group, (B) wild-type PPE group, (C) iNOS$^{-/-}$ control group, (D) iNOS$^{-/-}$ PPE group. (E) Total leukocytes. (F) ROS detection. Scale bar, 100 µm. Statistically different compared to control WT group, $^{*}p<0.05$, $^{**}p<0.01$, and statistically different compared to PPE WT group, $\#p<0.05$. $\#\#p<0.01$, $\#\#\#p<0.001$. $n=5$/group.

Table 4
Lung morphological and stereological quantifications in WT and iNOS$^{-/-}$ mice instilled or not with PPE.

	CTR WT	PPE WT	CTR iNOS$^{-/-}$	PPE iNOS$^{-/-}$
L_m (µm)	42.55 ± 1.41	77.36 ± 4.90**	41.96 ± 1.48$^{\#\#}$	49.01 ± 2.73
Sv (µm^{-1})	0.094 ± 0.003	0.053 ± 0.003**	0.096 ± 0.003$^{\#\#}$	0.083 ± 0.005
Vv[alveolar septa] (%)	61.7 ± 2.21	36.7 ± 2.34*	66.3 ± 1.60$^{\#\#\#}$	53.9 ± 3.18
Vv[air space] (%)	38.3 ± 2.21	63.3 ± 2.34*	33.7 ± 1.60$^{\#\#\#}$	46.1 ± 3.18

L_m, mean linear intercept length of distal air spaces; Sv, surface density of alveolar septa; Vv[alvsept], volume density of alveolar septa; Vv[air space], volume density of air spaces; PPE, porcine pancreatic elastase. The results represent the means ± SEM. $n=5$/group.

* $p<0.05$ vs wild-type control mice.
** $p<0.01$ vs wild-type control mice.
$^{\#\#}$ $p<0.01$ vs wild-type PPE mice.
$^{\#\#\#}$ $p<0.001$ vs wild-type PPE mice.

was observed at these time points. This finding is attributed to the actions of resident cells, such as alveolar epithelial cells or even resident macrophages, neutrophils, and lymphocytes, which initiated the inflammatory process by releasing the Th1 cytokine. Although these cells were not obviously increased in number, it is possible that they were more activated after PPE instillation. At the same time, a reduction in IL-10 cytokine levels was observed, indicating a proinflammatory profile with an increase in stimulatory cytokines and decrease in modulatory cytokines in the lung environment. Under normal physiological conditions, IL-10 inhibits the secretion of proinflammatory cytokines such as TNF-α and IFN-γ, suppressing the proliferation of T cells, NK cells, and alveolar macrophages [26]. However, under stimulatory conditions, IL-10 cannot carry out these immunomodulatory effects, favoring increased secretion of TNF-α and contributing to inflammation. Considering that few cells migrated to the alveolar spaces at 7 days, as shown in Fig. 2A, it is likely that the cells present were more active, i.e., they secreted more TNF-α. This rationale is consistent with the myeloperoxidase activity, which was increased beginning at 7 days after PPE instillation. Increased myeloperoxidase activity indicates the presence of neutrophil activity and is also a considerable aggravating factor for oxidative

stress because its reaction with H_2O_2 generates hypochlorous acid, which can react with the superoxide anion to form a hydroxyl radical, one of the strongest ROS [27]. It is surprising that variations in inflammatory mediators and oxidative stress markers occurred at 7 days after induction of emphysema, whereas the leukocytes in the BAL did not increase until 21 days, especially when at this time while the number of leukocytes was increasing, the markers TNF-α, IL-10, SOD, and TBARS had returned to control values. But inflammation first occurred in alveolar septa and later in the alveolar space. Inflammatory mediators and redox markers were assayed in lung homogenates, whereas leukocyte numbers were quantified in BAL. So, we suggest a temporal response between parenchyma and BAL that is proportional to the variation observed in our study. Thus TNF-α, IL-10, SOD, and TBARS were reduced in lung homogenates, because major leukocyte numbers were found at this time in the BAL.

The hydroxyproline profile is especially interesting. Hydroxyproline levels were elevated at 7 days, returned to baseline levels at 14 days, and increased again at 21 days after PPE instillation. The detection of OHpro in BAL is an indirect way to assess connective tissue/extracellular matrix breakdown [12,25]. Thus, the elevated levels of OHpro in BAL after PPE stimulus suggest that the breakdown of septal tissue was accelerated at specific time points, compromising the pulmonary architecture. This could be caused by protease release from inflammatory and epithelial cells or through the chemotactic action of peptides derived from the ECM, contributing to continuous tissue destruction [23].

The hydroxyproline response profile was paralleled by nitrite levels, which were increased at 7 days, reduced to control levels at 14 days, and increased again at 21 days after PPE instillation. This pattern of behavior suggests that a kinetic response is involved during the establishment of emphysema. Apparently, an initial response is triggered by PPE stimulation and associated effects, followed by a more stable stage featuring a "pseudo-effective" combating of the PPE effects and ending with a rebound elevation of specific markers during the emphysema phase. The NO involvement at various stages of emphysema suggests that it plays a pivotal role in the pathogenesis of emphysema and encouraged us to explore the underlying pathways. However, these data cannot be interpreted independently because NO is likely to interact with other molecules. Under physiological conditions, the superoxide anion is rapidly removed by high concentrations of scavenging enzymes called superoxide dismutases, and NO is rapidly removed by diffusion through tissues into red blood cells [28,29], where it is converted to nitrate/nitrite. However, under proinflammatory conditions, the production of superoxide and NO is simultaneously strongly activated, increasing the production of both molecules by 1000-fold [30]. These conditions favor the formation of the strong oxidant peroxynitrite, which is produced by the collision of NO and superoxide.

Elevated SOD and nitrite levels were observed at 7 days after PPE, indirectly indicating a parallel increase in O_2^- and NO production with consequent dismutation and conversion, respectively. In contrast, both were decreased at 14 days after PPE instillation, suggesting the involvement of peroxynitrite formation, with NO and superoxide reacting so quickly that SOD activity was not required. However, at 21 days, when emphysema was established, nitrite elevation was observed again but not accompanied by elevation in SOD activity, suggesting a possible failure of the antioxidant system and free peroxynitrite synthesis.

Oxidative stress is more evident at the initial phase of the establishment of emphysema than when the emphysema is fully developed. As shown by the time course after PPE instillation, SOD was elevated at 7 days; GPx was elevated at 7, 14, and 21 days; and TBARS, a recognized marker of oxidative damage by lipid peroxidation, was elevated at 7 and 14 days. These results clarify an important aspect of proteolytic emphysema, showing that oxidative stress is in fact involved in its establishment and progression. Additionally, these data highlight several parameters that should be considered markers of oxidative stress in proteolytic emphysema. Beyond the establishment of inflammation and oxidative stress conditions, PPE triggered additional interacting pathways. The stimulation of iNOS expression favors the enhanced production of NO, which is involved in inflammatory responses. These increases in NO levels could facilitate dangerous levels of peroxynitrite synthesis, which can react with tyrosine residues to form nitrotyrosine [22,31]. Because NO moves so readily through membranes and between cells, it can react with superoxide even if it has not been produced within the same cell [30]. To understand the participation of nitric oxide and its potential to react with superoxide anion to form peroxynitrite during the development of emphysema, animals were cotreated with aminoguanidine, a selective inhibitor of iNOS. The data from these experiments show that PPE induced nitration reactions, as demonstrated by the elevated nitrotyrosine expression, which is also observed in COPD patients [31]. Our data corroborate that of a recent study, which used another iNOS-selective inhibitor, L-NIL, to show that iNOS plays a crucial role in tobacco-smoke-induced emphysema [31]. Our data reinforce the critical role of iNOS activity during the development of proteolytic emphysema and highlight aminoguanidine as a good therapeutic strategy. A previous study from our group showed that an inhibitor (L-NAME) and a substrate (L-arginine) of NO synthesis have different effects on the emphysema caused by cigarette smoke, a source of oxidants [32]. The mechanism of action of the inhibitor used before is nonselective, because L-NAME is analogous to L-arginine, implying a competition for NO synthesis [33]. However, the mechanism of action of aminoguanidine is different because it acts preferentially on the inducible isoform of nitric oxide synthase [34]. Thus, a specific inhibition enables a better characterization of the pathway involved in the pathogenesis of emphysema. Nevertheless, in this study we used an elastase model of emphysema, which is not a source of oxidants, to prove that the oxidative and nitrosative pathways are involved in the process of induction of emphysema per se, regardless of the causative stimulus. The low iNOS expression observed in both groups treated with 1% AMG suggests that the inhibition of iNOS was incomplete. Nevertheless, 1% AMG effectively inhibited nitration and may also have modulated eNOS expression in response to PPE. According to Laszlo et al. [35], AMG is a dual constitutive and inducible nitric oxide synthase inhibitor. However, a review published in 2001 [36] compared different inhibitors of NOSs and showed that AMG has a partially selective action between iNOS and eNOS, being 11 times more selective for iNOS than for eNOS. Furthermore, they proposed a break in the paradigm, affirming that the activity of constitutive neuronal and endothelial NOSs can be regulated by various factors. Thus, we suggest that the proposed treatment (1% AMG) partially inhibited iNOS expression and avoided the overexpression of the constitutive isoform eNOS, slightly increased by PPE. Above all, treatment with 1% AMG improved the pulmonary histoarchitecture, preventing alveolar damage and inflammatory infiltration.

Compared to our study, a recent study published by Boyer et al. [37] showed contradictory results, concluding that neither eNOS nor iNOS is required for the development of elastase-induced emphysema. Although they found an increased number of 3-nitrotyrosine-positive cells in lung from PPE wild-type mice, they did not find any improvement in lung parenchyma of neither iNOS- or eNOS-deficient mice nor of mice treated with 1400W, an important iNOS-selective inhibitor. Perhaps these contradictory data are justified by crucial discrepancies between the two emphysema models. The previous one induced emphysema with a 10 times higher dose of PPE from elastin products compared to our study. Even with this elevated dose (5 U of PPE/mouse), our

results regarding the L_m parameter were more significant than those presented in that study ($p < 0.01$ and $p < 0.05$, respectively). Regarding the pharmacologic inhibition established with 1400W, it is possible that it achieved total iNOS inhibition because it is 30 times more selective for iNOS than for eNOS [38]. We suggest that 1% AMG partially inhibited iNOS, as we showed by Western blot. Maybe this modulation has a crucial role in the progression of proteolytic pulmonary emphysema. Surprisingly, our results from PPE iNOS$^{-/-}$ mice corroborate our previous data showing that iNOS-deficient mice instilled with PPE showed preservation of morphometric parameters such as L_m, Sv, and $V\nu$ of air space and septa and also avoided an increase in leukocyte numbers and ROS levels, which were increased in the PPE wild-type group. Thus, we suggest that a modulation of iNOS in both inflammatory and chronic processes such as COPD plays a fundamental role in attenuating its effects.

In conclusion, we have found a trio of pathways involved in the pathogenesis of pulmonary emphysema induced by proteolytic perturbation. Inflammation, oxidative stress, and nitrosative stress seem to be important variables that affect the development of emphysema. A limitation of our study was that we did not assay shorter time points of elastase stimulus, which could provide more information about the initial oxidative stress response to the development of emphysema. But one novel finding of this study is that oxidative stress plays a pivotal role at the initial phase of this disease. Nitrosative stress was observed at 21 days after PPE treatment, when emphysema had been fully installed, and aminoguanidine treatment concomitant with emphysema induction attenuated the proteolytic effects induced by PPE. Our results suggest that oxidative and nitrosative stresses generated by nitric oxide production via iNOS expression are critical in elastase-induced emphysema.

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