Inducible NOS Inhibition Reverses Tobacco-Smoke-Induced Emphysema and Pulmonary Hypertension in Mice

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SUMMARY

Chronic obstructive pulmonary disease (COPD) is one of the most common causes of death worldwide. We report in an emphysema model of mice chronically exposed to tobacco smoke that pulmonary vascular dysfunction, vascular remodeling, and pulmonary hypertension (PH) precede development of alveolar destruction. We provide evidence for a causative role of inducible nitric oxide synthase (iNOS) and peroxynitrite in this context. Mice lacking iNOS were protected against emphysema and PH. Treatment of wild-type mice with the iNOS inhibitor N6-(1-iminoethyl)-L-lysine (L-NIL) prevented structural and functional alterations of both the lung vasculature and alveoli and also reversed established disease. In chimeric mice lacking iNOS in bone marrow (BM)-derived cells, PH was dependent on iNOS from BM-derived cells, whereas emphysema development was dependent on iNOS from non-BM-derived cells. Similar regulatory and structural alterations as seen in mouse lungs were found in lung tissue from humans with end-stage COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD), which includes both chronic bronchitis and emphysema, is expected to be ranked as the third-greatest cause of death worldwide by 2020 (Murray and Lopez, 1997). One pathological concept suggests that COPD develops through airway inflammation and remodeling. The main theory behind emphysema development is the destruction of the elastic architecture of the lung, leading to enlargement of distal air spaces (Black et al., 2008). Moreover, COPD/emphysema is increasingly viewed as a systemic disease, involving skeletal muscle wasting, diaphragmatic dysfunction, and systemic inflammation (Agustí et al., 2003).

An estimated 30%–70% of patients with COPD also have pulmonary hypertension (PH); however, there is much debate about the numbers of patients affected by PH, and many patients with COPD have no severe PH (Minai et al., 2010). Thus, the relevance of a vascular pathology for the pathogenesis of COPD is still unresolved. PH was often thought to occur as a consequence of the hypoxia associated with COPD, but there is increasing evidence that tobacco smoke may have a direct impact on the pulmonary vasculature (Peinado et al., 2008), indicating that cor pulmonale and late-stage PH are not necessarily secondary to hypoxia in patients with COPD. These data are supported by studies in guinea pigs, showing that a vascular phenotype can precede parameters of emphysema development (Ferrer et al., 2009; Wright and Churg, 1990, 1991). However, to the best of our knowledge, no published study directly compares the course of emphysema development and PH in other species.

Oxidative and nitrosative stress, chronic inflammation, apoptosis, and altered proliferation have been suggested as factors in the pathogenesis of airway remodeling (Churg et al., 2008; Ricciardolo et al., 2004; Stockley et al., 2009; Tsoumakidou et al., 2005). This has led to attention being focused on the involvement of interleukins, the vascular endothelial-derived growth factor (VEGF) system, matrix metalloproteinases (Mmp), and reactive oxygen species (ROS) (Taraseviciene-Stewart and Voelkel, 2008; Yoshida and Tuder, 2007) in destroying the lung architecture. ROS and reactive nitrogen species have long been known to cause protein modification and DNA damage (Wink and Mitchell, 1998). Indeed, nitric oxide (NO) reacts with superoxide (O2−) to form the potent oxidant peroxynitrite (ONOO−) (Szabó et al., 2007); this in turn can react with tyrosine residues to form nitrotyrosine, the levels of which are increased in COPD (Ricciardolo et al., 2004; Tsoumakidou et al., 2005).
NO is synthesized from L-arginine by nitric oxide synthase (NOS), which exists as three isoforms (Moncada and Erusalimsky, 2002). Increased NO production and nitrosative stress in COPD may derive from enhanced expression or activity of inducible NOS (iNOS) and endothelial NOS (eNOS) (Brindicci et al., 2009). However, the regulation of NOS isoforms during the course of emphysema development has not yet been addressed.

In order to investigate the molecular mechanisms involved in disease progression as a basis to develop new strategies to treat COPD, we aimed to (1) decipher the role of NOS in the development of emphysema and examine possible vascular alterations leading to PH after tobacco-smoke exposure and (2) investigate a possible link between vascular alterations and emphysema development.

RESULTS

Pulmonary Hypertension Precedes Lung Emphysema Development in Wild-Type Mice Exposed to Tobacco Smoke

Exposure of wild-type (WT) mice to tobacco smoke for up to 8 months resulted in the development of lung emphysema after 6 months, as evident from an increase in the mean linear intercept, an increase in the air space, and a decrease in the septal wall thickness (Figures 1A–1C).

Within 3 months, tobacco-smoke exposure caused increases in right ventricular systolic pressure and the ratio of the absolute numbers of alveoli to the number of vessels, followed by right-heart hypertrophy (Figures 1D–1F): i.e., development of PH preceded the development of lung emphysema. PH was associated with an increase in the degree of muscularization in the pulmonary arteries (diameter 50–100 μm) (Figures 1G and 1H). A similar increase in the degree of muscularization was found in larger pulmonary arteries (data not shown). The late onset of lung emphysema development compared with the development of PH was also evident from lung-function data (Figure S1A).

Alterations Caused by Tobacco-Smoke Exposure

Within 3 months, tobacco-smoke exposure caused increases in right ventricular systolic pressure and the ratio of the absolute numbers of alveoli to the number of vessels, followed by right-heart hypertrophy (Figures 1D–1F): i.e., development of PH preceded the development of lung emphysema. PH was associated with an increase in the degree of muscularization in the pulmonary arteries (diameter 50–100 μm) (Figures 1G and 1H). A similar increase in the degree of muscularization was found in larger pulmonary arteries (data not shown). The late onset of lung emphysema development compared with the development of PH was also evident from lung-function data (Figure S1A).

We compared the development of PH and emphysema after 8 months of tobacco-smoke exposure in iNOS−/−, enOS−/−, and WT mice. The iNOS−/− mice were protected against the development of emphysema and PH, as evident from quantification of (1) mean linear intercept, air space, septal wall thickness (Figures 3A–3C); (2) right ventricular systolic pressure, the ratio of alveolus/vessels, right-heart hypertrophy (Figures 3D–3F); and (3) the degree of muscularization (Figure 3G). By contrast, eNOS−/− mice developed emphysema and PH to the same degree as WT controls (Figures 3A–3G). Similar patterns were also seen in lung-function parameters (Figure S3A).

iNOS Inhibition Prevented Smoke-Induced Functional Deterioration and Reversed Deterioration of Fully Established Emphysema

Treatment of WT mice with the iNOS-selective inhibitor L-NIL was started in parallel to smoke exposure and resulted in protection against the development of lung emphysema, as shown by alveolar morphology (Figures 3H–3J), and against PH, as shown by hemodynamic measurements and morphometry (Figures 3K–3N). These findings were mirrored by lung-function parameters (Figure S3B).

Lung structure and function were restored when WT mice were treated with L-NIL in a curative approach after full establishment of emphysema (i.e., initiation of L-NIL treatment after 8 months of chronic smoke exposure for an additional 3 month period) (Figures 3H–3N). Lung regeneration did not occur in placebo-treated animals. Analysis of lungs from a second, independent set of curatively L-NIL-treated mice revealed (besides effects on cellular components; Figures S3C and S3D) that the number of alveoli (assessed by stereological morphometry, which excludes lung-volume-dependent effects) was significantly reduced after 8 months of smoke exposure and restored by curative L-NIL treatment but not by placebo treatment (Figure 4Aii). In vivo lung-function assessment from these animals revealed an increase in lung dynamic compliance after 8 months of smoke exposure, which was reversed upon curative L-NIL treatment but not with placebo (Figure 4Aii). The reversal effect of L-NIL treatment was also evident for PH (Figure 4Aiii). Detailed investigation and quantification of elastic-fiber structure by light,
confocal, and electron microscopy showed destruction of elastic fibers during tobacco-smoke exposure and regeneration upon curative L-NIL treatment (Figures 4B–4D and S4).

**Cellular Components of PH and Emphysema Development**

A detailed fluorescence-activated cell sorter analysis performed using the BAL and the remaining lung homogenate showed a tendency toward increased numbers of granulocytes, macrophages, activated macrophages, CD4+ T cells (and CD8+ T cells, but these were generally detected in very low numbers), and activated T cells in the homogenate after 3 and 8 months of smoke exposure. In the BAL, this trend only became evident for activated granulocytes after 8 months of smoke exposure (Figure S3C). Interestingly, curative L-NIL treatment significantly downregulated the number of granulocytes, macrophages, activated macrophages, and T cells (and also tended to activate T cells) in the homogenate. In contrast, no such downregulation was detected in the BAL (Figure S3C). Combined quantification of macrophages from both the alveolar and nonalveolar compartments by immunohistochemistry confirmed an upregulation of those cells and a reduction upon curative L-NIL treatment (Figure S3D).
To further decipher the role of iNOS in bone marrow (BM)-derived versus non-BM-derived cells for the development of emphysema and PH, we generated chimeric mice, where BM from iNOS−/− mice was transplanted into WT mice (iNOS−/− → WT) and vice versa (WT → iNOS−/−). WT-to-WT BM transplantation served as a control. Quantification of alveolar numbers by stereology, in vivo hemodynamics and lung function, and vascular morphometry showed that WT-to-WT transplanted mice developed emphysema and PH after 4 months of smoke exposure. Whereas the WT → iNOS−/− mice were protected from emphysema, iNOS−/− → WT mice were not, as seen from the changes in the number of alveoli as well as in vivo compliance (Figures 5A and 5B). In contrast, only those chimeric mice with deletion of iNOS in the BM cells were protected from vascular alterations and PH (Figures 5C and 5D).

Molecular Pathways Explaining Lung Emphysema Development and Its Reversal upon Curative L-NIL Treatment

ONOO− is a possible candidate for mediating the effects of iNOS upregulation on lung vasculature and parenchyma. As downstream signaling of ONOO− can be mediated via nitrotyrosine formation, we investigated nitrotyrosine levels in WT, eNOS−/−, iNOS−/−, and L-NIL-treated mice, confirming a key role of iNOS for nitrotyrosine formation (Figures 6A and S5A). We found that ONOO− indeed caused nitration in primary isolated alveolar epithelial cells, pulmonary vascular endothelial cells, and pulmonary arterial smooth muscle cells from resistance vessels (data not shown). Subsequently, apoptosis was found to be induced in epithelial and endothelial cells. Proliferation, however, was only reduced in epithelial cells. No significant effect was found

Figure 2. Localization and Relative Quantification of iNOS and eNOS in Wild-Type Mouse Lungs

(A) Immunostaining (i and ii; red) and nonisotopic in situ hybridization (iii and iv; green) for iNOS and eNOS in WT mouse lung sections. V = vessel, B = bronchus. (B) Quantitative real-time polymerase chain reaction analysis for iNOS (i) and eNOS (ii) mRNA of small pulmonary vessels (outer diameter 50–100 μm), septa, and bronchi (outer diameter 140–300 μm). iNOS and eNOS values were related to porphobilinogen deaminase mRNA levels. (i and ii) Data are from duplicate measurements of n = 20 vessels from n = 3 lungs each. (C) Western blot analysis of iNOS (i) and eNOS (ii) from lung homogenate, normalized to β-actin. Values are from measurements of n = 6 individual lungs each. Data are given for 3 and 8 months of tobacco-smoke exposure and for unexposed controls (0 months). A representative blot is shown on the right and densitometry is given on the left. Data are presented as mean ± SEM. *Significant difference (p < 0.05) compared with unexposed controls (i.e., 0 months of exposure). See also Figure S2.
on apoptosis or proliferation in pulmonary arterial smooth muscle cells (Figures 6B–6C). As a possible downstream link, we investigated key mediators of proliferation and apoptosis, like c-Jun N-terminal kinase (JNK), Src, and ERK phosphorylation. p-JNK was upregulated in alveolar epithelial and pulmonary vascular endothelial cells (Figure 6D), but p-ERK and p-Src levels were not affected by OONO−/C0 in any cell types investigated, and JNK, ERK, and Src inhibitors could not antagonize OONO−/C0 effects on proliferation and apoptosis (not shown). Investigation of the effects of OONO− on Rtp801 and VEGF formation revealed that the Rtp801 protein was upregulated only in epithelial cells, and VEGF was downregulated in epithelial and pulmonary arterial smooth muscle cells (Figures 6E and 6F).

In addition, we addressed mechanisms of apoptosis, proliferation, extracellular matrix destruction/restoration, oxidative stress, and inflammation in small pulmonary artery vessels (diameter 50–100 μm), alveolar septa, and small bronchi (diameter 140–300 μm; Figure S5B). This revealed that apoptosis markers were consistently upregulated in the vascular compartment by 3 months of smoke exposure, whereas no such upregulation occurred in the alveolar septa. Importantly, this upregulation of apoptosis markers in the vascular compartment was strongly counter-regulated by curative L-NIL treatment, but no such effects were seen in the alveolar or bronchial compartments (Figure S5B). L-NIL treatment attenuated or reversed the downregulation of the majority of the cell proliferation markers (Figure S5B). This finding was supported by proliferating-cell nuclear antigen (PCNA) staining (Figure S5C). In addition, several genes found to be regulated by curative L-NIL treatment for the categories of extracellular matrix regulation, oxidative stress, and inflammation correlated with the restorative effects of such treatment (Figure S5B).
Determination of O$_2$ related to a per-cell basis (Figures S2A and S2B). The increase. Conversely, NO levels from BAL cells were unchanged increased in the homogenized lungs, reflecting iNOS upregula-

(A) Total number of alveoli assessed by quantitative stereology (i), in vivo dynamic lung compliance (ii), and right ventricular systolic pressure (iii); n = 5 each.
(B) Quantification of elastin by determination of the amount of intact elastic fibers (i) and elastin score (ii) via light microscopy from paraffin-embedded lungs (n = 5 each) or elastin content in cryopreserved lungs (iii) by confocal microscopy (CM) (n = 3).
(C) Representative slides of elastin-stained lungs from CM.
(D) Elastic fibers (*) magnified by electron microscopy.

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Comparison of Human End-Stage COPD in Smokers
to a Mouse Model of Emphysema Induced
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When comparing lung tissue from patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV COPD (Rabe et al., 2007) and a history of smoking (Table S1) to that of healthy donors, we found a similar increase in mean linear intercept and air space measures and a similar decrease in septal wall thickness (Figures 7A–7C and 7F) as seen in WT mice after 8 months of tobacco-smoke exposure. In addition, similar to mice, an increase in the ratio of the number of alveoli to the number of vessels and an increased degree of vessel muscularization were found in the tissue samples from patients with COPD (Figures 7D–7F) compared to healthy donors. Smokers who had not yet developed COPD displayed similar vascular

**Figure 4. Pulmonary Effects of iNOS Inhibition in Wild-Type Mice following Chronic Smoke Exposure**

(A) Total number of alveoli assessed by quantitative stereology (i), in vivo dynamic lung compliance (ii), and right ventricular systolic pressure (iii); n = 5 each.
(B) Quantification of elastin by determination of the amount of intact elastic fibers (i) and elastin score (ii) via light microscopy from paraffin-embedded lungs (n = 5 each) or elastin content in cryopreserved lungs (iii) by confocal microscopy (CM) (n = 3).
(C) Representative slides of elastin-stained lungs from CM.
(D) Elastic fibers (*) magnified by electron microscopy.

Data are presented as mean ± SEM. *Significant difference (p < 0.05) between groups as indicated. See also Figure S4.
alterations to those observed in smokers suffering from COPD (Figures 7A–7I). Analysis of iNOS and eNOS expression in human lung tissue also showed a similar pattern of regulation in the COPD lungs and in those of donor controls as in smoke-exposed and control mice. In circumstances where iNOS was upregulated in the pulmonary vasculature, eNOS was downregulated (Figures 7G and 7H). Levels of nitrotyrosine protein in the pulmonary vasculature and alveoli were increased in samples from patients with COPD compared with donor controls (Figure 7I). Again, similar regulation profiles were found in smokers without COPD.

DISCUSSION

We sought to determine whether the vascular pathology is linked to emphysema development and identified iNOS as a key molecular player in the underlying processes. Our data showed that alterations in lung vascular structure and function induced by tobacco smoke preceded emphysema in mice and were independent of hypoxia. We also showed that emphysema and PH occurred independently and are essentially associated with iNOS in different cell types. Finally, our data indicate that targeting iNOS by pharmacological inhibition can improve the functional and structural destruction caused by tobacco smoke.

Long-term exposure to tobacco smoke in our mouse model enabled analysis of the temporal order of structural and functional changes in both the pulmonary vasculature and the airways and alveolar structures during the development of lung emphysema. The development of lung emphysema in our model correlated with previous reports that used mice exposed to tobacco smoke (Churg et al., 2008). Vascular remodeling upon tobacco-smoke exposure has been shown in humans (Peinado et al., 2008) and in animal models (Ferrer et al., 2009; Wright and Churg, 1991; Wright et al., 2006, 2011); however, we provide here a detailed direct comparison of alterations in vascular morphology and function and their temporal relationship to alveolar destruction in mice. In this regard, dysregulation of iNOS and nitrotyrosine formation have been proposed as underlying mechanisms of COPD (Brindicci et al., 2009).
Our investigations of the cellular contribution to both emphysema development and PH in chimeric mice showed that both can occur independently. The complete dependency of pulmonary vascular alterations on BM-derived iNOS-containing cells can be explained by a derivation of a portion of pulmonary vascular cells from BM cells. It has been suggested that BM-derived cells contribute to pulmonary vascular remodeling, and that pulmonary arterial smooth muscle cells may be generated from BM-derived cells (Asosingh et al., 2008; Huertas and Palange, 2011). Indeed, the importance of BM cell-derived iNOS for induction of systemic vascular disease was highlighted recently by Ponnuswamy and colleagues (Ponnuswamy et al., 2009). Alternatively, iNOS from non-smooth muscle, BM-derived cells could contribute also to pulmonary vascular remodeling. The fact that emphysema was only prevented in chimeric mice that lacked iNOS in non-BM-derived cells excludes an essential role for iNOS in the pathogenesis of emphysema in macrophages or other BM-derived cells. This supports the concept of a prominent role of non-BM-cell-derived iNOS and thus, e.g., vascular iNOS for emphysema development. Interestingly,
the data from the chimeric mice further suggested that emphysema development can be dependent on vascular iNOS in the absence of vascular remodeling. The fact that emphysema development and PH in the chimeric mice were triggered by different cell populations and, thus, can occur independently may explain why only a portion of patients suffering from COPD develop PH (if the mice data are transferable to the human situation).

The increased levels of nitrotyrosine, present as a possible consequence of ONOO− generation (Szabó et al., 2007), in WT mice following tobacco-smoke exposure were in accordance with our hypothesis that ONOO− upregulation is a key step in vascular remodeling and emphysema pathogenesis. Interestingly, ONOO− both induced apoptosis and reduced proliferation in alveolar epithelial cells but caused only apoptosis in endothelial cells. This result agreed with the observed effects on alveolar and vascular pruning in our animal studies. Such effects of ONOO− are also concordant with previous findings of apoptosis induction (Szabó et al., 2007), a mechanism important for the development of lung emphysema (Yoshida and Tuder, 2007).

Figure 7. Comparison of the Alveolar and Vascular Structure, eNOS and iNOS Expression, and Nitrotyrosine Formation in Lungs from Human Patients with Severe COPD, Smokers without COPD, and Healthy Donor Controls

(A–C) Alveolar morphometry given as (A) mean linear intercept, (B) air space, (C) septal wall thickness.

(D) Ratio of the number of alveoli to the number of vessels.

(E) Degree of muscularization of small pulmonary arteries (diameter 20–70 µm). Data are given as percentage of total vessel count for fully muscularized (Full), partially muscularized (Partial), and nonmuscularized (None) vessels.

(F) Representative histology from stained lung sections, representing alveolar changes (i) or antibodies against α-smooth muscle actin (violet) and von Willebrand factor (brown = endothelial cell marker) (ii).

(G) iNOS (i) and eNOS (ii) immunostaining. V = vessel.

(H) Expression of iNOS mRNA (i), iNOS protein (ii), eNOS mRNA (iii), and eNOS protein (iv).

(i) Densitometric data from a western blot analysis of nitrotyrosine from homogenized lung tissue.

*Significant difference (p < 0.05) compared with healthy donor controls. Data are derived from human GOLD stage IV COPD lungs (smoker+COPD), smokers without COPD (smoker−COPD), and healthy donor control lungs (donor) and are presented as mean ± SEM. See also Table S1.
ONOO$^-$ upregulated Rtp801, a protein identified as essential for emphysema development in mice (Yoshida et al., 2010), in alveolar epithelial cells but not endothelial or vascular smooth muscle cells. Such a regulation has been suggested to involve downstream VEGF inhibition, leading to alveolar epithelial cell apoptosis (Ellisen, 2010; Yoshida et al., 2010). VEGF gene knockout causes emphysema in mice (Taraseviciene-Stewart and Voelkel, 2008), and VEGF has been shown to be essential for lung growth and maintenance (Voelkel et al., 2006). In accordance, we showed downregulation of VEGF in alveolar epithelial type II cells (AECII) and primary murine pulmonary arterial smooth muscle cells (PASMC) upon ONOO$^-$ challenge.

Analyzing gene-regulatory processes upon curative L-NIL treatment revealed several candidates that can be linked to lung regeneration or support regeneration by attenuation of disease progression. L-NIL reversed the upregulation of Mmp9 (a marker of parenchyma destruction), and the mRNA of the metalloproteinase inhibitor encoded by Timp3 was upregulated in the vascular compartment with L-NIL treatment. It has previously been shown that Timp3 knockout mice develop emphysema (Leco et al., 2001). The reversal of the mRNA downregulation of pro-proliferative factors like Fgf10 and Ccnr1 in alveolar septa by curative L-NIL treatment correlates with the increase in PCNA-positive cells. Corroborating these findings, the upregulation of mRNA of proapoptotic genes Bax, Tnfsf10, Fosb, Traf1, and Fastk in the vascular compartment was again antagonized by curative L-NIL treatment.

Our detailed analysis of the elastic fiber structure of the lung supports the concept that the increase in proliferation upon curative L-NIL treatment is part of an active restructuring process of the lung. Different methods of quantification and ultrafine structural images revealed that the amount, as well as the structure, of elastin fibers is reduced and degraded, respectively, during tobacco-smoke exposure and is substantially reversed upon curative L-NIL treatment.

Interestingly, curative L-NIL treatment selectively reduced the number of granulocytes, macrophages, activated macrophages, and T cells in the lung-tissue compartment. Downregulation of macrophage numbers may coincide with reduced O$_2^-$ production, leading to decreased oxidative stress and thus reduced ONOO$^-$ levels, but also to reduced inflammatory mediator levels (Chung and Adcock, 2008). In addition, the observed downregulation of T cells correlates with the hypothesis that autoimmune mechanisms may contribute to COPD development (Churg et al., 2008; Feghali-Bostwick et al., 2008; Motz et al., 2010). Moreover, it is suggested that T cells are involved not only in emphysema development but also in the pathological remodeling of the pulmonary vasculature (Austin et al., 2010; Cutoffa et al., 2011). As our data from the chimeric mice show that the entire vascular remodeling process is dependent on BM-derived iNOS-expressing cells, this effect could be due either to inflammatory cells like macrophages or to iNOS-containing stem cells. Moreover, data from our experiments performed in chimeric mice showed that emphysema development can be independent of iNOS in BM-derived cells and, thus, is at least independent from iNOS in macrophages, activated macrophages, granulocytes, T cells, and activated T cells.

Examination of lung tissue from ten patients with severe COPD (GOLD stage IV) who had undergone lung transplantation revealed an upregulation of iNOS (mRNA and protein), increases in nitrotyrosine content, and alterations in vascular and alveolar structure and function qualitatively similar to those seen in the smoke-exposed WT mice. In addition, we have corroborated previous findings in lung-tissue samples from smokers who had not developed COPD, demonstrating vascular alterations in direct association with tobacco-smoke exposure (Peinado et al., 2008). The similar profiles of iNOS and nitrotyrosine regulation in smokers with COPD and smokers who had not developed COPD suggest that portions of the pathways deciphered in our mouse model also may have an impact on human COPD; however, this conclusion has to be drawn cautiously, as our data are for a limited number of patients, are for end-stage COPD, and are heterogeneous in nature when compared with the mouse lung data. In addition, a confounding impact of hypoxia or treatment including steroids should be taken into account when applying the mouse data to the human situation. If iNOS inhibition is to be investigated further as a clinical means of treatment for emphysema, one has to consider possible side effects related to disruption of iNOS function. As iNOS is expressed in many cell types, including nonimmune cells and immune cells (Bogdan, 2001), several cellular and systemic, including immune, functions may be disrupted by iNOS inhibition. However, to date, no adverse effects concerning increased susceptibility to infections have been reported in clinical studies with selective iNOS inhibitors (Brindicci et al., 2009; Singh et al., 2007). Owing to its effects on T cell regulation, it has been suggested that abrogation of iNOS function could potentially exacerbate autoimmune diseases such as colitis, arthritis, or multiple sclerosis (Niedbala et al., 2007). There are also implications for iNOS playing a role in systemic vascular disease, and this has been assessed in animal models including atherosclerosis (Porrunswamy et al., 2008). Thus, given the potentially wide-ranging effects of iNOS inhibition, any clinical intervention for emphysema may require local application of iNOS inhibitors via inhalation or cell-type-specific targeting of iNOS inhibition.

In conclusion, our study highlights that the effects of tobacco smoke on the pulmonary circulation preclude the development of alveolar destruction and emphysema formation, and both vascular and alveolar changes occur in an iNOS-dependent manner in mice. Furthermore, emphysema and PH development are not essentially linked but can occur independently. If transferable to humans, these findings could explain the hitherto controversial discussion about the impact of PH on emphysema development in humans, where PH is not always associated with emphysema. Finally, we suggest that selective iNOS inhibition offers the potential to reverse emphysema.

**EXPERIMENTAL PROCEDURES**

**Animals**

Adult male WT C57BL/6J, iNOS$^{-/-}$, and eNOS$^{-/-}$ (B6.129P2-Nos2tm1Lau/J and B6.129P2-Nos3tm1va/J) mice, 20–22 g, were obtained from Charles River Laboratories, Sulzfeld, Germany. Animals were housed under controlled conditions with a 12 hr light/dark cycle and food and water supply ad libitum. Animals were randomly allocated to tobacco-smoke-exposed and -unexposed groups of six mice each, with parallel groups for (1) alveolar...
mice were exposed to tobacco smoke for 8 months with parallel application 6 hr/day, 5 days/week for up to 8 months. For preventive treatment, WT of 3R4F cigarettes (Lexington, KY, USA) at 140 mg particulate matter/m³ for 2007). After incubation with ONOO⁻, cells were cultured in serum-free medium overnight prior to ONOO⁻ application. Proliferation assay was performed as described previously (Mittal et al., 2007). After incubation with ONOO⁻, the culture medium was replaced with regular medium, containing serum and [³²P]thymidine (Amersham, Munich, Germany). After incubation at 37°C and 5% CO₂ for 4 hr, cells were harvested and [³²P]thymidine incorporation was measured by liquid scintillation spectrometry.

For assessment of apoptosis, the CaspACE Assay System, Colorimetric (Promega, Mannheim, Germany), was used according to the manufacturer’s instructions. Additionally, 1 µM staurosporine (Sigma-Aldrich, Munich, Germany) for 4 hr was used as a positive control.

VEGF-ELISA
For detection of VEGF in cell culture medium, the RayBio Mouse VEGF ELISA Kit (RayBiotech, Inc.) was used. Experiments were performed according to the protocol provided by the supplier.

Generation of Bone Marrow-Transplanted Chimeric Mice
Generation of chimeric mice was performed as previously described (Voswinckel et al., 2003) with modifications. For details, refer to Extended Experimental Procedures online.

Alveoli Count via Design-Based Stereology
For counting of alveoli, uniform random sampling and the physical dissector method were used as described previously (Ochs et al., 2004). For details, refer to Extended Experimental Procedures online.

Localization of eNOS, iNOS, and Nitrotyrosine
Localization of eNOS and iNOS was investigated in lung sections from cryopreserved tissue by immunostaining, as described previously (Mittal et al., 2007). Nitrotyrosine was detected in paraffin-embedded lung sections of both mouse and human lung tissue using a rabbit anti-nitrotyrosine antibody (Sigma-Aldrich). For details, refer to Extended Experimental Procedures online.

PCNA Staining
For immunohistochemical localization of the proliferation marker PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the AP-fast red kit (Zytochem, Berlin, Germany) was used on paraffin-embedded lung sections according to the manufacturer’s instructions. All stained sections were analyzed using digital slide scanning employing a mirax scanner and the mirax viewer software (Carl Zeiss GmbH, Jena, Germany).

Nonisotopic In Situ Hybridization
Localization of mRNA by nonisotopic in situ hybridization (NISH) was determined in cryostat lung sections as previously described (Mittal et al., 2007). For details, refer to Extended Experimental Procedures online.

Laser-Assisted Microdissection
Laser-assisted microdissection (LMD 6000, Leica, Nußloch, Germany) was performed to isolate pulmonary arterial vessels, bronchi, and septa from cryostat lung section as previously described (Mittal et al., 2007). For details, refer to Extended Experimental Procedures online.

RNA isolation, Pre amplification, cDNA Synthesis, and Real-Time PCR
RNA from laser-microdissected or homogenized mouse and human lung tissue was isolated by RNeasy Micro and Mini kits, respectively (QIAGEN, Hilden, Germany). The isolated RNA was converted to cDNA, and relative quantification of the eNOS and iNOS mRNA was performed using the iQ SYBR Green Supermix (BioRad, Munich, Germany). For details, refer to Extended Experimental Procedures online.

Real-Time PCR-Based PCR Array
Vessels (diameter 50–100 µm), septa, and bronchi (diameter 140–300 µm) were laser-microdissected from 8 µm sections of Tissue Tek-embedded mouse lungs fixed after BAL. PCR-based arrays were performed using customized 96-well plates containing primers for selected genes, according to the manufacturer’s instructions (SA Biosciences/Biominol, Hamburg, Germany).
Western Blot
For the quantification of eNOS, iNOS, and nitrotyrosine in mouse and human lung tissues, the polyclonal antibodies anti-eNOS (BD Biosciences, Heidelberg, Germany), or anti-iNOS (Abcam, Cambridge, UK), raised in rabbits, and anti-nitrotyrosine (Abcam, Cambridge, UK), raised in mice, were used. For the quantification of (phospho)-SAPK/JNK, (phospho)-ERK (both Cell Signaling, Danvers, MA, USA), (phospho)-Src (Epitomics, Burlingame, CA, USA), and Rfp801 (Abnova, Heidelberg, Germany) in mouse PASMC, EC, and AEGII, the respective polyclonal antibodies raised in rabbits were used. For details, refer to Extended Experimental Procedures online.

Quantification of Lung Elastin by Image Analysis
Analysis was performed as previously published with modifications (Bigatel et al., 1999; Black et al., 2008; Lawrence et al., 2004). For details, refer to Extended Experimental Procedures online.

Confocal Microscopy and Quantification of Elastin Immunolabeling
Experiments were performed on cryosections of 30 μm thickness using specific antibodies against elastin and α-smooth muscle actin. For details, refer to Extended Experimental Procedures online.

Transmission Electron Microscopy
The tissue was fixed in 3% glutaraldehyde and embedded in Epon following routine procedures. Ultrathin sections were double stained with uranyl acetate and lead citrate and viewed in a Philips CM 10 or a CM 201 electron microscope (Philips, Andover, MA, USA).

Patient Characteristics
Human lung tissues were obtained from transplanted COPD patients (GOLD stage IV). Smokers without COPD, and donor controls. The patients’ characteristics are given in Table S1. The studies were approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (AZ 31/93), Giessen, Germany.

For all other experimental procedures, please refer to the Extended Experimental Procedures online.

Statistical Analyses
Comparison of multiple groups was performed by analysis of variance (ANOVA) with the Student-Newman-Keuls post-test. If several groups were compared to one control, an ANOVA with Dunnett’s test was performed. For comparison of two groups, a Student’s t test was performed. We considered p values below 0.05 as statistically significant for all analyses.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, five figures, and one table and can be found with this article online at doi: 10.1016/j.cell.2011.08.035.

REFERENCES


