Overexpression of the Serine Elastase Inhibitor Elafin Protects Transgenic Mice From Hypoxic Pulmonary Hypertension

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Background—Increased serine elastase activity has been implicated in the vascular remodeling associated with chronic hypoxia-related pulmonary hypertension in rats.

Methods and Results—In this study we determined the time course of hypoxia-induced serine elastase activity in the murine lung and related this to initiation of a proteolytic cascade characterized by an increase in matrix metalloproteinases (MMPs). We then used transgenic mice in which overexpression of the selective serine elastase inhibitor elafin was targeted to the cardiovascular system to determine whether upregulation of a naturally occurring serine elastase inhibitor suppresses MMPs and the hemodynamic and structural response to chronic hypoxia (air at 380 mm Hg). In nontransgenic but not in elafin-transgenic mice, we documented a transient increase in serine elastase activity after 12 hours of hypoxic exposure attributed to a 30-kDa protein as determined by elastin zymography and fluorophosphonate/fluorophosphate-biotin labeling. Two days after hypoxia, the pro-forms of MMP-2 and MMP-9 were induced in the nontransgenic mice, but MMP-9 was suppressed in elafin-transgenic mice. Acute hypoxic vasoconstriction was similar in nontransgenic and elafin-transgenic littermates. Chronic hypoxia for 26 days resulted in >1-fold increase in right ventricular pressure (P<0.004) in nontransgenic compared with control or elafin-transgenic littermates. In the latter mice, normalization of the right ventricular pressure was associated with reduced muscularization and preservation of the number of distal vessels (P<0.04 for both comparisons).

Conclusions—Modulation of the severity of chronic hypoxia-induced pulmonary vascular disease could be a function of endogenously expressed serine elastase inhibitors. (Circulation. 2002;105:516-521.)

Key Words: hypoxia • hypertension, pulmonary • lung

Pulmonary hypertension occurs in a variety of clinical conditions and can be reproduced experimentally in mice by exposure to hypobaric hypoxia. After acute hypoxia-induced vasoconstriction, the progression to chronic pulmonary hypertension is reflected in elevation in right ventricular (RV) pressure and in RV hypertrophy and is attributed to the structural remodeling of pulmonary arteries. These include abnormal muscularization of distal vessels owing to differentiation of precursor pericytes to smooth muscle cells (SMCs) as well as medial hypertrophy of muscular arteries. Loss of arteries is also documented in hypoxia-exposed rats.

We previously reported an increase in endogenous vascular elastase activity in the pulmonary arteries of rats exposed to hypobaric hypoxia. Serine elastases degrade extracellular matrix (ECM), release growth factors, and result in increased deposition of ECM proteins such as tenasin, which amplify the proliferative response to growth factors. Degraded matrix peptides also induce matrix metalloproteinases (MMPs), which could further contribute to the pathology. In this way, serine elastase activity could initiate a cascade that results in SMC proliferation and medial thickening of the distal pulmonary arteries, leading to progressive pulmonary hypertension in response to chronic hypoxia. Our previous studies have shown that intravenous or oral administration of serine elastase inhibitors reduces the severity of chronic hypoxia-related pulmonary hypertension and vascular disease in rats.

We created a transgenic mouse in which the specific serine elastase inhibitor elafin was targeted to the cardiovascular system, expressed in the heart, lungs, and arteries, and was functionally active. These mice exhibited reduced SMC proliferation and medial/intimal thickening after carotid artery wire injury. In this study we established the nature and
time course of elevated serine elastase and MMP activity after hypoxia exposure in mice and assessed whether, in elafin-transgenic mice, the intrinsic ability to regulate the endogenous levels of a serine elastase inhibitor might modulate the extent of hypoxia-induced pulmonary vascular disease.

Methods

Experimental Design

Randomly assigned CD1, elafin-transgenic, and nontransgenic littermates were exposed to hypoxia for up to 40 days in a hypobaric chamber (air at 380 mm Hg), were kept in room air, or were exposed to hypoxia for only 12 hours followed by a 21-day recovery in room air. Mice were genotyped as previously described.13 We identified 77 transgenic and 75 nontransgenic mice from 23 litters used in this study. Although this resulted in differences in the number of animals in each group, it also ensured that mice of matched genetic backgrounds and age were being compared.

Proteolytic Activity

Elastase Activity

Mice were euthanized with phenobarbital (20 mg/kg), a thoracotomy was performed, and lungs were perfused with PBS through the main pulmonary artery to remove the blood. Elastase activity was extracted from tissues as previously described.16 The elastase assay was performed with the use of DQ-elastin substrate, as described by the supplier ( Molecular Probes). In some samples, the general serine proteinase inhibitor PMSF (2 mmol/L), the selective serine elastase inhibitor elafin (50 μg/mL), or the metalloproteinase inhibitor EDTA (5 mmol/L) was added before DQ-elastin. For comparisons between room air and 48 hours after hypoxia, the elastase assay was performed with the use of a fluorescent substrate, AFC091 (40 μmol/L; Enzyme Systems), as previously described.11

Fluorophosphonate/Fluorophosphate-Biotin Labeling

Extracts prepared for the elastase activity assays were preincubated with avidin agarose (Sigma) for 1 hour at 4°C. Fluorophosphonate/fluorophosphate (FP)-biotin17 was then added to a final concentration of 0.3 μmol/L, and samples were incubated at 25°C for 1 hour, then subjected to SDS-PAGE. The gel was transferred to a nitrocellulose membrane, and FP-biotin–labeled serine proteinase bands were visualized with the use of avidin–horseradish peroxidase (HRP) conjugate (BioRad), as previously described.17

Gelatinase Activity

Lungs were homogenized in 0.9% NaCl and centrifuged; the supernatants were assayed with DQ-gelatin substrate ( Molecular Probes).

Zymography

Samples were electrophoresed onto 7.5% PCR-SDS gels containing 1 mg/mL of soluble elastin (Elastin Products) or gelatin (Sigma) substrates. SDS was removed from the gels by shaking in 2.5% Triton-X100 for 1 hour. The gels were then rinsed in water and incubated for 24 to 36 hours at 37°C in 50 mmol/L Tris-Cl, pH7.4, 150 mmol/L NaCl and 5 mmol/L CaCl2. Elastolytic bands were visualized by Coomassie staining. For elastin or gelatin substrate gels, samples were prepared as described above for the activity assays. Densitometric quantification of the protease bands was performed using a Gel Documentation System (BioRad).

Northern Blot for Endothelin-1 mRNA

Total RNA was isolated from the lungs with Trizol (Life Technologies). RNA was then electrophoresed on formaldehyde-agarose gels, transferred to Hybond N+-membranes (Amersham-Pharmacia, Piscataway, NJ), and hybridized to the radiolabeled human endothelin-1 cDNA (2.1 kb; American Type Culture Collection, Manassas, Va).

Assessment of RV Pressure

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/mL, MTC Pharmaceuticals) and xylazine (10 mg/kg, Bayer) and then orotracheally intubated with a 22-gauge polyethylene catheter and ventilated under controlled pressure (10 cm H2O) at 100 respirations per minute. Under a surgical microscope, the chest was opened with a midline sternotomy, and the right superior vena cava was identified. A small incision was made distal to a hemostatic ligature, and a 1.4F Millar microcatheter (model SPR671) was advanced into the RV. Pressure tracings were acquired on a Pentium-II computer with customized software (P. Baackx, University of Toronto) and analyzed with the use of Origin-5.0 (Microcal).

RV Hypertrophy and Morphometric Analysis of Pulmonary Arteries

The lungs were cleared of blood as described above, the trachea was ligated with a second catheter, and the heart and lungs were removed en bloc. Tissues were fixed by perfusion with 10% formalin. The RV free wall was separated from the left ventricle and septum and weighed on an analytical balance. Transverse sections of the left lung were stained with van Gieson elastin stain, and vessels (15- to 50-μm external diameter) were counted. All morphometric analyses were performed by one observer blinded as to the type of mouse from which the tissue was taken. In each lung section, the ratio of arteries to alveoli was quantified from three randomly chosen fields at ×10 magnification, and an average value was calculated.

Immunostaining

To specifically distinguish SMCs in assessing muscularization of distal vessels, lung sections were immunostained with HRP-conjugated undiluted anti–SMC-α–actin monoclonal antibody (DAKO), as previously described.11 HRP-conjugated mouse IgG was used as a negative control. Sections were then stained with hematoxylin. In each lung section, vessels of 15- to 50-μm external diameter were identified as associated with an alveolar duct or an alveolar wall and were classified as fully muscularized (actin staining >75% of the circumference), partially muscularized (actin staining 25% to 75% of the circumference), or nonmuscularized (<25%). In each lung section, the percentage of alveolar duct or wall arteries fully or partially muscularized was calculated.

Statistical Analysis

All data are presented as mean±SEM. Statistical significance was evaluated by ANOVA, and post hoc comparisons were made with the Fisher’s protected least significant difference test.

Results

Serine Elastase Activity After Hypobaric Hypoxia

We subjected CD1 mice to hypoxia for 3, 6, 12, 24, and 48 hours to assess the duration and magnitude of elastase activity. After a 3-hour exposure to hypoxia, elastase activity was actually reduced by ≈36% compared with normoxia controls (P<0.010). Values returned to control levels at 6 hours and were increased by ≈47% at 12 hours (P<0.001). This was not sustained at 24 hours (Figure 1A) or 48 hours (data not shown). At 7, 14, and 21 days of hypoxic exposure, elastase activity was reduced compared with normoxia (P<0.001).

The heightened elastolytic activity at 12 hours was characterized by incubating the extracts with inhibitors before the assay. Complete inhibition of elastolytic activity was seen with the nonselective serine proteinase inhibitor PMSF or the more selective serine elastase inhibitor elafin. There was no reduction in enzymatic activity with the metalloproteinase inhibitor EDTA (Figure 1B). A single ≈27-kDa elastolytic
band was observed by zymography on an elastin substrate gel, the activity of which was abolished when samples were preincubated with PMSF or elafin but not EDTA (Figure 1C).

To determine the molecular weight of the serine elastase in mouse lung, extracts were incubated with FP-biotin, which reacts with the active site residues of serine proteinases. A band of ≈30 kDa was identified (Figure 1D). The reaction of FP-biotin with the serine elastase from lung was completely blocked when the samples were preincubated with the serine elastase inhibitor elafin.

We next compared the elastase activity of lungs of nontransgenic and elafin-transgenic littermates subjected to hypoxia or room air for 12 hours. In nontransgenic mice, we confirmed a 1-fold increase in elastase activity after a 12-hour hypoxia exposure (P<0.002) but not in the lungs of elafin-transgenic mice, in which elastase activity was not significantly different from normoxia controls.

Expression of MMPs
Increase in elastase activity results in degradation of the ECM and release of growth factors, which induce MMPs both at the mRNA and protein levels. Increased MMP-9 activity is associated with ECM degradation in lungs of rabbits after hypoxia. In the lungs of nontransgenic mice hypoxic for 48 hours, induction of the pro-form of MMP-9 was observed by gelatin zymography but not in the lungs of elafin-transgenic mice (Figure 2). Pro-MMP-2 levels were significantly elevated in both nontransgenic and elafin-transgenic hypoxic littermates, with only a trend toward a reduction in elafin-transgenic mice (P=0.059). With the use of the DQ-gelatin substrate, however, no significant differences were observed in total gelatinase activity reflecting active MMPs at this point.

Endothelin-1 mRNA Expression
Because the preproendothelin promoter was used to target elafin-transgene expression to the cardiovascular system, we examined the possibility that this might have interfered with the endogenous expression of endothelin-1 mRNA. We therefore performed Northern blot analyses but found no significant differences in endothelin-1 mRNA expression comparing elafin-transgenic and nontransgenic littermates in room air or hypoxia for 48 hours (n=4 in each group) (data not shown).

RV Pressure
We next determined whether inhibition of elastase activity and MMP expression in elafin-transgenic mice was protective...
against the chronic pulmonary hypertension associated with hypoxia-induced structural remodeling of pulmonary arteries. RV pressures were examined in mice subjected to room air, acute hypoxia (10% oxygen for 15 minutes), and chronic hypoxia (air at 380 mm Hg) for 26 days (Figure 3). No significant differences were found in the acute hypoxic vasoconstrictor response, but chronic hypoxia for 26 days produced a significant increase in RV pressure in nontransgenic mice compared with both elafin-transgenic littermates and normoxia controls \( (P<0.004) \) in which values were similar.

Hematocrit and RV Hypertrophy

A similar increase in the hematocrit and in RV hypertrophy (RV/body weight and RV/LV+S ratios) was observed in elafin-transgenic and nontransgenic littermates subjected to chronic hypoxia for 26 and 40 days (Table). Only a modest decrease in RV/LV+S ratio was observed at 10 days after hypoxia in elafin-transgenic compared with nontransgenic mice subjected to hypoxia and normoxia controls \( (P<0.05) \).

**Muscularization and Loss of Pulmonary Vessels of 15- to 50-μm Diameter**

To assess the extent of muscularization in the pulmonary vessels, lung sections from nontransgenic and elafin-transgenic mice that were subjected to hypoxia or room air for 26 days were immunostained for SMC α-actin (Figure 4A). A \( >4 \)-fold increase in the percentage of fully and partially muscularized alveolar duct vessels was seen in nontransgenic mice subjected to 26 days of chronic hypoxia versus normoxic controls \( (P<0.004) \). This feature correlated with RV pressures \( (R=0.787, P=0.011) \) and was repressed in elafin-transgenic compared with nontransgenic hypoxic littermates \( (P<0.04) \). In fact, values were not different from those in normoxic controls \( (P>0.09) \). No significant differences were, however, observed in the population of muscularized alveolar wall arteries comparing elafin-transgenic and nontransgenic littermates. A 41% reduction in the number of arteries of 15- to 50-μm external diameters relative to alveoli was observed in the lung sections of nontransgenic hypoxic versus normoxic mice \( (P<0.001) \). In elafin-transgenic hypoxic littermates, the number of peripheral arteries relative to alveoli was similar to that observed in normoxic controls (Figure 5). Owing to the transient nature of the elevation in serine elastase activity, we carried out subsequent experiments to address whether only a 12-hour exposure to hypoxia followed by recovery in room air for 21 days would be sufficient to induce the vascular remodeling described above. No significant differences were observed compared with room air controls.

**Discussion**

We demonstrated a transient early increase in serine elastase activity in the lungs that was completely inhibited in the elafin-transgenic mice. The heightened elastase activity in nontransgenic mice after hypoxia was associated with elevated MMP-9 as well as increased muscularity and loss of distal arteries leading to the rise in RV pressure, whereas in elafin-transgenic mice these features were repressed. Al-
Figure 4. Muscularization of distal pulmonary arteries in mice hypoxic for 26 days. Lung sections were immunostained for elastin. A, Fully muscularized artery associated with alveolar duct of a nontransgenic hypoxic mouse. Arrows denote alveolar duct–associated arteries. Morphometric analyses of fully plus partially muscularized pulmonary arteries (15- to 50-µm external diameter) associated with alveolar ducts. Normoxia: n=4 for nontransgenic; n=3 for elafin-transgenic. Hypoxia (26 days): n=8 for nontransgenic; n=8 for elafin-transgenic. Bars represent mean±SEM. *P<0.004 vs normoxia. †P=0.035 vs nontransgenic hypoxia.

Figure 5. Loss of distal pulmonary arteries in mice subjected to chronic hypoxia for 26 days. Lung sections were stained with van Gieson elastin stain; muscularity was assessed as described in Methods. A, Fully muscularized artery associated with alveolar duct of a nontransgenic hypoxic mouse. Arrows denote alveolar duct–associated arteries. B, Morphometric analyses of fully plus partially muscularized pulmonary arteries (15- to 50-µm external diameter) associated with alveolar ducts. Normoxia: n=4 for nontransgenic; n=3 for elafin-transgenic. Hypoxia (26 days): n=8 for nontransgenic; n=8 for elafin-transgenic. Bars represent mean±SEM. *P<0.02 vs normoxia. †P<0.02 vs nontransgenic hypoxia.

MMP expression can be increased by serine elastases either through release of degraded matrix peptides or growth factors such as FGF-2 or by inhibition of tissue inhibitors of MMPs. In fact, at 2 days after hypoxia, an elevation in the pro-forms of both MMP-2 and MMP-9 was observed. It is possible that elevation in gelatinase activity (active MMPs) may have occurred at a later time point. Elevation of active MMPs has been observed in the lungs of CD-1 mice (our unpublished observations) and rabbits after hypoxia.

There was no elevation in endothelin mRNA levels at 48 hours of hypoxia in the lungs of nontransgenic or elafin-transgenic littermates versus normoxia controls. This contrasts with reports in rats in which induction of endothelin appears to be critical for the acute hypoxic vasoconstriction and subsequent vascular remodeling. This difference in hypoxia-induced regulation in the mouse versus the rat suggests alterations in the structure of the endothelin gene or in the factors with which it interacts. This result, however, confirms that targeting elafin expression to the cardiovascular system by the preproendothelin promoter does not interfere with endogenous endothelin expression.

Consistent with a similar vascular phenotype in elafin-overexpressing mice and their control littermates, the functional response is comparable because acute hypoxia results in equivalent vasoconstriction. The hemodynamic response to chronic hypoxia was, however, quite different and attributable to elafin-mediated suppression of the early transient elevation in serine elastase activity. The reduced RV pressure in elafin-transgenic mice compared with control littermates was related to suppression of both muscularization and loss of peripheral arteries. Muscularization of the precapillary arterioles is expected to result in increased pulmonary resistance leading to elevation in the RV pressure as would numerical restriction of the pulmonary vascular bed owing to loss of distal arteries. Although the reduction in the severity of the
Structural changes correlated with a decrease in RV pressure, there was no evidence that RV hypertrophy was suppressed. This is probably a result of chronic elevation in hematocrit, continuous hypoxic vasoconstriction, and direct effects of hypoxia on the myocardium.

We propose that muscularization of peripheral arteries is related to elastase-mediated proliferation and differentiation of pericytes to SMCs. The elafin-mediated reduction in muscularization of distal vessels was evident in the alveolar duct rather than the more peripheral alveolar wall component of the pulmonary circulation that was affected primarily in hypoxia-exposed rats treated with elastase inhibitors. Thus, it is likely that the reduced alveolar duct arterial muscularity in conjunction with the restoration of the number of distal vessels was responsible for the amelioration of RV pressure.

There may be several reasons why the loss of peripheral pulmonary vessels was less severe in the elafin-transgenic mice. It is possible that in response to hypoxia, endothelial swelling and elastase-mediated degradation of basement membrane glycoproteins and proteoglycans cause closure and resorption of some of the distal arteries or that the increased muscularity of more proximal arteries leads to their closure.

Our study therefore provides proof of concept that endogenous levels of serine elastase inhibitors might determine, at least in part, the structural and functional response to a pulmonary hypertension producing stimulus, thereby regulating the severity of pulmonary vascular disease and accounting for individual variability.

Acknowledgments

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References


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