Decreased Neprilysin and Pulmonary Vascular Remodeling in Chronic Obstructive Pulmonary Disease

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Rationale: Studies with genetically engineered mice showed that decreased expression of the transmembrane peptidase neprilysin (NEP) increases susceptibility to hypoxic pulmonary vascular remodeling and hypertension; in hypoxic wild-type mice, expression is decreased early in distal pulmonary arteries, where prominent vascular remodeling occurs. Therefore, in humans with smoke- and hypoxia-induced vascular remodeling, as in chronic obstructive pulmonary disease (COPD), pulmonary activity/expression of NEP may likewise be decreased.

Objectives: To test whether NEP activity and expression are reduced in COPD lungs and pulmonary arterial smooth muscle cells (SMCs) exposed to cigarette smoke extract or hypoxia and begin to investigate mechanisms involved.

Methods: Control and advanced COPD lung lysates (n = 13–14) were analyzed for NEP activity and protein and mRNA expression. As a control, dipeptidyl peptidase IV activity was analyzed. Lung sections were assessed for vascular remodeling and oxidant damage. Human pulmonary arterial SMCs were exposed to cigarette smoke extract, hypoxia, or H₂O₂, and incubated with antioxidants or lysosomal/proteasomal inhibitors.

Measurements and Main Results: COPD lungs demonstrated areas of vascular rarefaction, distal muscularization, and variable intimal and prominent medial/adventitial thickening. NEP activity was reduced by 76%; NEP protein expression was decreased in alveolar walls and distal vessels; mRNA expression was also decreased. In SMCs exposed to cigarette smoke extract, hypoxia, and H₂O₂, NEP activity and expression were also reduced. Reactive oxygen species inactivated NEP activity; NEP protein degradation appeared to be substantially induced.

Conclusions: Mechanisms responsible for reduced NEP activity and protein expression include oxidative reactions and protein degradation. Maintaining or increasing lung NEP may protect against pulmonary vascular remodeling in response to chronic smoke and hypoxia.

Keywords: pulmonary hypertension; smooth muscle cell; smoking; oxidative stress; protein degradation

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Pulmonary vascular remodeling is an important contributor to pulmonary hypertension due to chronic smoke inhalation and hypoxia, as in chronic obstructive pulmonary disease (COPD). In mice, decreased activity/expression of the transmembrane peptidase neprilysin increases hypoxic pulmonary hypertension, but the involvement of neprilysin in human secondary pulmonary vascular disease has not been investigated.

What This Study Adds to the Field

We show that neprilysin activity and expression are substantially decreased in human lungs with advanced COPD and in isolated human pulmonary arterial smooth muscle cells exposed to cigarette smoke extract and hypoxia, by mechanisms that include oxidative reactions and protein degradation. The results suggest that increasing lung neprilysin could be a useful strategy to treat or prevent vascular remodeling and pulmonary hypertension complicating chronic lung disease.

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Chronic obstructive pulmonary disease (COPD) is a leading cause of death; cigarette smoking is its number one risk factor (1). Pulmonary vascular remodeling, characterized by thickening, muscularization, and rarefaction of the distal vasculature (2–4), complicates COPD by contributing to pulmonary hypertension (PHTN) (3, 5). Many patients with COPD have mild PHTN at rest (6–8); however, the prevalence of exercise-induced PHTN, which also may lead to right-sided heart failure in COPD (9), is much higher (> 91% [6, 10]).

COPD-associated PHTN is likely caused by initial injury of the pulmonary vascular endothelium by cigarette smoke (CS) (4), followed by inflammation and hypoxia, all of which may involve oxidant mechanisms (11, 12). Factors that may contribute to variable susceptibility to COPD-associated PHTN, including interleukin-6 (IL-6) and the serotonin transporter (5HTT) (6, 10, 13, 14), have been extensively investigated.

Neprilysin (NEP, CD10) is a transmembrane zinc peptidase that degrades specific peptides and is widely expressed, including in pulmonary arterial (PA) smooth muscle cells (SMCs), endothelial cells, and fibroblasts (15). NEP activity/expression is decreased by CS (16), hypoxia (17, 18), or reactive oxygen species (ROS) (19). Hypoxic NEP null mice develop greater PHTN, and PA SMCs from these mice grow faster than those from wild-type mice, suggesting that NEP protects against chronic hypoxic PHTN, in part by suppressing PA SMC growth and migration (17). However, NEP's role in human pulmonary

vascular remodeling complicating chronic lung disease has not been investigated.

As a control, dipeptidyl peptidase IV (DPPIV, CD26) was analyzed; this peptidase is also widely expressed, including in the pulmonary vasculature. Its structure is similar to that of NEP (20, 21); the two enzymes share some substrates, but others are unique (22–24). Vasoactive intestinal peptide (VIP), a potent pulmonary vasodilator with antiinflammatory and antiproliferative effects (25), is metabolized more by DPPIV than NEP (22). In contrast to NEP, DPPIV inhibition may promote lung function (26). DPPIV's involvement in COPD or PHTN is unknown.

We investigated whether a reduction in NEP occurs (versus DPPIV) in human lungs with smoke- and hypoxia-induced vascular remodeling, similar to that observed in hypoxic mice (17). Advanced COPD lungs were obtained from patients with FEV₁ less than 30%, as worsening PHTN has been associated with decreased FEV₁ (8, 27). We measured NEP activity, protein, and mRNA in human lung samples and PA SMCs exposed to CS extract (CSE), hypoxia, and hydrogen peroxide (H₂O₂). Further mechanisms relevant to COPD-associated PHTN, including oxidant effects on NEP activity and protein degradative changes, were studied in human PA SMCs.

Our findings suggest that lung NEP levels may be predictive of susceptibility to pulmonary vascular remodeling and PHTN in COPD, and that higher levels may protect against vascular injury induced by chronic CS and hypoxia. Our results, some of which have been reported in the form of abstracts (28–30), may lead to novel preventions, tests, and treatments.

METHODS

See the online supplement for a more detailed version of these methods.

Human Lung Tissue

Control and COPD (FEV₁% of predicted > 80 and < 30, respectively) frozen lungs and slides were from the University of Colorado Denver COPD Center, National Institutes of Health (NIH) Lung Tissue Research Consortium (LTRC; www.ltrcpublic.com), or the Interstitial Lung Disease Program, National Jewish Health, Denver (NJH). Sample processing conformed to LTRC guidelines. Samples in RNA later were from the LTRC and NJH. Sections were reviewed by a lung pathologist to verify designations. Studies on de-identified human tissue were approved by the Colorado Multiple Institution Review Board (COMIRB), exemption #07–0791.

Immunohistochemistry

Control or COPD sections (4 μ m) were fixed in 10% formalin and embedded in paraffin at the UCD, LTRC, or NJH sites (Table 1). Staining (17) was with anti-human NEP, nitrotyrosine, CD31 (PECAM-1), or α -smooth muscle (SM) actin; counterstain was hematoxylin.

Pulmonary Vascular Remodeling and NEP Scoring

Distal (25–100 μ m) PA (five per lung) were scored blindly on a scale of 0 to 3+ by a lung pathologist for intimal, medial, and adventitial structural changes on hematoxylin and eosin-stained slides. Additional morphometric analysis (31) was performed on pentachrome-stained slides (32). Density of distal PAs was measured in CD31-stained slides. For NEP-stained slides, intensity in the alveolar walls and distal vessels (nine areas per lung) was scored blindly on a scale of 0 to 4+ (17).

Western and Activity Analyses

Lung samples or PA SMCs were homogenized in 20 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5, containing protease inhibitors, and for PA SMCs, 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate. NEP activity was measured as described (17). DPPIV activity was measured by the DPPIV-Glo Protease Assay

(Promega, Madison, WI). Western analyses with anti-human NEP were assessed by densitometry. Routine examination of Coomassie Blue-stained blots assured sample integrity, equal loading, and transfer.

Transcriptional Analysis

DNase-treated RNA was isolated with the RNeasy kit (Qiagen, Valencia, CA). mRNA was semiquantitated (33) with real-time polymerase chain reaction. Reactions (25 μ l), containing 0.25 μ g cDNA, 0.1 μ M primers, and iQ SYBR Green Supermix (BioRad, Hercules, CA), were amplified by 40 cycles of 15 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C. NEP primers were from Qiagen (QT00048755); reference was β -actin.

Inactivation of NEP Activity by H₂O₂

Incubations of human rNEP with varying concentrations of H₂O₂, or of control human lung homogenates with 100 μ M H₂O₂, were conducted for 24 hours at 37°C, diluted 40- to 60-fold, and assayed for remaining NEP activity (19).

Human PA SMCs

Human PA SMCs (main PA) were from Clonetics/Lonza (Walkersville, MD). Cells were made quiescent with 0.1% fetal bovine serum and exposed to 5 μ g/ml CSE, hypoxia (3% O₂), or 100 μ M H₂O₂ at 37°C for 4 or 48 hours. Antioxidants (tiron [2.5 mM], MnTMPyp [a superoxide dismutase (SOD)/catalase mimetic (34), 20 μ M], or polyethylene glycol [PEG]-catalase [40 U/ml]) or protein degradation inhibitors (Folimyacin [35] for lysosomes, 50 nM; Clasto-lactacystin β -lactone [36] for proteasomes, 1 μ M) were added 0.5 or 2 hours, respectively, before exposures.

Statistical Analyses

Data are mean $\pm$ SEM. Group sizes needed were determined with PASS 2008 (NCSS, Kaysville, UT). Statistical significance ($P < 0.05$) was determined by *t* test, one-way, or two-way analysis of variance, as appropriate (JMP, SAS Institute, Cary, NC) (17).

RESULTS

Human Lung Tissue

Control lung tissue from tissue donors and lung lobectomies/wedge resections was from the University of Colorado Denver COPD Center, the Interstitial Lung Disease Program (NJH), and the NIH LTRC. Advanced COPD lung tissue from transplants was from the UC Denver COPD Center and the NIH LTRC. Sources and characteristics of control and COPD lung samples are listed in Table 1. Clinical sites were designated “Denver” or “near sea level,” because Denver altitude (5,280 ft) impacts PaO₂ measurements and may subtly alter vascular structure compared with the other procurement sites. The average age of the control group ($n = 14$) was 57.5 ± 4.7 years, and that of the advanced COPD group ($n = 13$) was 51.0 ± 1.6 years. The control group contained 9 women and 5 men, 3 of whom were smokers and 11 of whom were nonsmokers. The advanced COPD group contained 7 women and 6 men, of whom 11 were smokers, 1 was a nonsmoker, and 1 had unknown smoking status. Some pulmonary function data were available for control patients from the LTRC, and for all the patients with advanced COPD undergoing lung transplantation. Those control subjects who were tested had averages of $106.0 \pm 3.2\%$ for forced expiratory volume in 1 second (FEV₁), $103.0 \pm 4.5\%$ for diffusing capacity of carbon monoxide (DL_{CO}), and 100.0 ± 0 mm Hg for PaO₂. The advanced COPD group had averages of $19.3 \pm 1.2\%$ for FEV₁, $31.3 \pm 5.6\%$ for DL_{CO}, and 58.3 ± 3.8 mm Hg for PaO₂. No hemodynamic data were available for the LTRC patients, and therefore this endpoint was not included in the analysis.

TABLE 1. SOURCE AND RELEVANT PROPERTIES (IF KNOWN) OF HUMAN LUNG SAMPLES

Source	Sample No.	Age, yr	Sex	Smoker	Pack-years	Clinical Site	FEV ₁ (% of predicted)	D _{LCO} (% of predicted)	RA PaO ₂ (mm Hg)*	Type of Sample
Controls										
UCD	Normal 9	22	M	N	0	Denver	—	—	—	D
UCD	Normal 10	17	F	N	0	Denver	—	—	—	D
UCD	Normal 11	71	F	N	0	Denver	—	—	—	D
UCD	Normal 12	78	F	Y	—	Denver	—	—	—	D
UCD	Normal 14	69	M	N	0	Denver	—	—	—	D
NJH	NL 65	51	M	N	0	Denver	—	—	—	D
NJH	NL 78	51	F	N	0	Denver	—	—	—	D
NJH	NL 79	65	F	N	0	Denver	—	—	—	D
NJH	NL 84	60	M	N	0	Denver	—	—	—	D
LTRC	289351	54	F	N	0	Near sea level	114	103	100	L/W
LTRC	222391	72	F	N	0	Near sea level	112	86	100	L/W
LTRC	203575	62	F	N	0	Near sea level	100	115	—	L/W
LTRC	036842	46	M	Y	30	Near sea level	84	72	100	L/W
LTRC	110594	55	F	Y	5	Near sea level	106	103	100	L/W
Advanced COPD										
UCD	164, SO2- 7591F	59	F	Y	40	Denver	20	43	66	T
UCD	183, S04-2570H	51	M	N	0	Denver	20	18	53	T
UCD	188, S04-4179E	66	M	Y	80	Denver	15	20	41	T
UCD	192, S04-5280	62	F	Y	30	Denver	16	19	60	T
LTRC	157421	46	M	—	—	Near sea level	20	—	—	T
LTRC	122541	55	F	Y	37	Denver	19	—	—	T
LTRC	294945	52	F	Y	1	Near sea level	15	—	80	T
LTRC	192361	50	F	Y	70	Near sea level	12	—	50	T
LTRC	219183	45	M	Y	20	Denver	17	59	—	T
LTRC	012861	51	M	Y	116	Near sea level	22	33	—	T
LTRC	295167	49	M	Y	36	Near sea level	27	—	—	T
LTRC	132625	63	F	Y	120	Near sea level	24	17	—	T
LTRC	224471	60	F	Y	9	Denver	21	16	—	T

Definition of abbreviations: D = donor, unused lung tissue; D_{LCO} diffusing capacity of carbon monoxide; F = female; FEV₁ = forced expiratory volume in 1 second; LTRC = National Institutes of Health Lung Tissue Research Consortium; L/W = lung lobectomy/wedge resection; M = male; N = no; NJH = Interstitial Lung Disease (ILD) Program, National Jewish Health; RA = room air; T = lung transplant; UCD = University of Colorado Denver COPD Center; Y = yes.

Control lung tissue from UCD and NJH were from tissue donors with no history of lung disease; those from the LTRC were taken from uninvolved lung tissue during lobectomy/wedge resection for malignant and benign lesions. Slides were examined by a lung pathologist to verify correct classification.

* PaO₂ measurements were obtained on RA at Denver altitude or near sea level (University of Michigan, University of Pittsburgh, or Mayo Clinic).

Analysis of Vascular Remodeling in Control versus COPD Human Lungs

To screen for rarification of the distal lung vasculature, we determined the density of distal PAs in control and advanced COPD lung sections. Lung sections were stained with an antibody for CD31, which stains endothelial cells. There were more CD31-positive distal, small (25–100 μ m) pulmonary blood vessels per square millimeter in control lung sections than in comparably inflated areas of emphysematous lung in the advanced COPD sections, indicating that there had been dropout or rarification of the distal lung vasculature in the COPD samples (Figure 1A). Even in these advanced COPD samples, patchy areas of relatively normal lung parenchyma and vascular density were encountered near areas of emphysematous lung. To evaluate remodeling of the pulmonary vessel wall, hematoxylin and eosin-stained lung sections were scored on a scale from 0 to 3+ by a blinded lung pathologist for relative thickening of the intima, media, and adventitia of distal (25–100 μ m) PAs (0 equals normal and 1, 2, and 3+ represent progressively more substantial thickening). As shown in Figure 1B, the COPD lung sections displayed progressively more thickening of the intimal, medial, and adventitial layers of the distal pulmonary vessels (25–100 μ m) compared with control samples. Additional distal vascular wall measurements using MicroBrighfield digital image analysis of pentachrome-stained sections corroborated these findings, although the magnitude of the differences was less (not shown). Examination of intermediate vessels (100–500 μ m) demonstrated that there was also pulmonary vascular remodeling at this site in COPD lungs, with intimal changes being more

prominent here. Medial and adventitial differences versus control were again noted but were not as substantial (not shown).

Figures 1C–1H show representative images of intermediate (100–500 μ m) and distal (25–100 μ m) PAs stained with pentachrome or with antibodies to CD31 or α -SM actin. Pentachrome staining of control versus advanced COPD lungs demonstrates intimal thickening (especially in the intermediate vessels), expansion of the medial layer between the internal and external elastic lamina, and prominent adventitia with loose yellow staining of matrix protein beyond the external elastic lamina. CD31 staining highlights the endothelium; note the lack of intimal change in the vessel shown (Figure 1E), demonstrating the variable remodeling observed at that site. α -SM actin stain demonstrates medial and adjacent adventitial thickening in advanced COPD lungs.

NEP Activity and Expression in Control and COPD Lung

We first assayed control and advanced COPD lungs for changes in NEP activity and expression. As shown in Figure 2A, there was a 76% decrease in NEP activity between control and COPD human lung lysates. The activity of another peptidase also found within the lung, DPPIV, measured for comparison, displayed no differences, between control and COPD lung (P = not significant; Figure 2B). Results of Western analyses, summarized in Figure 3A, demonstrate a 48% decrease in NEP protein expression between control and COPD samples. Representative NEP-stained Western blots and Coomassie Blue-stained nitrocellulose membranes are shown in Figure 3B.

brown: 3,3'-diaminobenzidine [DAB]). Note the absence of intimal changes in this vessel, an example of the variability in this signal. (H) α -smooth muscle (SM) actin-stained distal PA of COPD sample (media is brown: DAB). Note the prominent adjacent adventitia. Small, distal vessels are 25–100 μ m. Larger, intermediate vessels are 100–500 μ m. Small arrowheads (D and E) point to the intimal (endothelial) vessel layer. Small arrows (D, E, G, and H) point to the medial (smooth muscle cell) vessel layer. Large arrowhead (H) points to the adventitial vessel layer. Black scale bars = 50 μ m; striped scale bars = 100 μ m.

Coomassie Blue staining was routinely used as a control for our Western blots, as well as for a check on sample and transfer integrity (37, 38). We could not detect DPPIV by Western analyses, despite using five different primary antibodies ($n = 16$). Next, NEP mRNA levels were semiquantitated in whole lung samples (stored in RNAlater) by quantitative real-time polymerase chain reaction. As shown in Figure 3C, a trend to an approximately 30% decrease in relative NEP mRNA level was observed between control and COPD samples ($P =$ not significant).

Immunostaining with an NEP antibody, which has been well characterized (17), was used to localize the expression of NEP in the lung. In preliminary serial dilution studies, alveolar walls and distal vessels (25–100 μ m) were noted to retain the strongest signal; more proximal structures had less staining than we had previously observed in mice (17). Proximal structures in slides of peripheral human lung are really intermediate in size and thus are different than those encountered in sections of mouse lung. NEP expression was widely decreased in COPD versus control lungs. The biggest differences appeared to be within alveolar walls and distal vessels, so these were studied in more detail; a 1:100 dilution of antibody was selected for comparative analysis. Control and COPD samples ($n = 5$ slides each) were evaluated in nine different areas of the slide. The intensity of NEP stain in the alveolar walls and distal vessels was scored in a blinded fashion, on a scale from 0 to 4+. As seen graphically in Figure 4A, in control lungs, NEP is expressed

prominently in alveolar walls and distal PAs, and expression does not vary significantly between these two areas in control lungs (2.32 ± 0.09 vs. 2.15 ± 0.18). However, in the COPD samples, NEP expression is decreased compared with control samples, in both the alveolar walls and distal vessels. In the COPD lungs, NEP expression may vary between these two areas; NEP expression is particularly low in the distal vasculature of the COPD lungs (the site of the greatest pulmonary vascular remodeling in the COPD lungs; COPD alveolar walls = 0.78 ± 0.16 ; COPD vessels = 0.16 ± 0.08). Figures 4B–4C show representative images of alveolar walls and adjacent distal vessels from slides of control and COPD lung tissue stained with anti-NEP antibody. Preliminary examination of NEP-stained lungs from subjects with other causes of PHTN not associated with substantial parenchymal disease (including idiopathic and collagen vascular disease-associated PHTN, $n = 6$), suggested also that distal remodeled pulmonary vessels had decreased levels of NEP (not shown).

Consistent with the peptidase activity measurements, immunohistochemical analyses for DPPIV using two primary antibodies with different levels of stringency confirmed DPPIV protein expression was unchanged in control versus COPD lungs ($n = 12$; not shown).

Finally, we looked for potential differences in NEP activity/expression and smoking history in our control and advanced COPD groups. Among the three control smokers, NEP activity

Figure 1. Increased vascular remodeling in chronic obstructive pulmonary disease (COPD) versus control lungs. (A and B) Morphometric analysis. (A) Endothelial stain demonstrates rarification in areas of emphysematous lung. Lung slides were stained with an antibody to the endothelial marker, CD31. The number of CD31-positive small pulmonary arteries (25–100 μ m) per unit area were counted in 10 similarly inflated areas per sample and averaged. $n = 4$ lung samples for control or COPD. (B) Human lung sample slides were stained with hematoxylin and eosin and reviewed by a blinded lung pathologist. The three layers (intima, media, adventitia) of the small pulmonary arteries (25–100 μ m) in each sample were scored from 0 to 3+ for structural changes (i.e., increased thickness). Five arteries were analyzed from each lung slide and the score was averaged for each lung. $n = 7$ lung samples for control or COPD. * $P < 0.05$ compared with control. (C–H), Representative immunohistological images demonstrating prominent pulmonary vascular remodeling in COPD samples. (C, D, and F, G) Pentachrome-stained intermediate and distal pulmonary arteries (PAs) (gray: endothelial; pink: medial; and yellow: adventitial layers are shown; C and F are control; D and G are COPD). (E) CD31-stained intermediate PA in COPD sample (endothelium is

Figure 2. Neprilysin (NEP), but not dipeptidyl peptidase IV (DPPIV), activity is reduced in lung lysates from patients with chronic obstructive pulmonary disease (COPD) with pulmonary vascular remodeling. (A) NEP activity was determined fluorometrically, $n = 13$ – 14 per group, $*P < 0.017$ compared with control (0.05/3, upon application of Bonferroni correction for multiple comparisons). (B) DPPIV activity was determined by luminescence. $n = 4$ – 5 per group.

and protein expression were reduced about 30% versus the highest control values, but NEP mRNA levels were normal. Nonsmokers or light smokers with advanced COPD with confirmed (183, SO4 2570H) or suspected (294945 and 224471) α_1 -antitrypsin deficiency had decreases in NEP activity and expression that were consistent with what was observed with the heavier smokers within the advanced COPD group. Thus, CS probably can inhibit NEP activity and protein expression, but CS might not decrease NEP mRNA. A subtle primary or secondary smoke exposure history, increased genetic susceptibility, and ongoing inflammation and parenchymal destruction may be associated with decreases in NEP activity and both NEP protein and mRNA expression.

Nitrotyrosine Staining of Control and COPD Lungs

To determine whether lungs from patients with advanced COPD are under increased oxidant stress, we stained control and advanced COPD lung sections for nitrotyrosine residues (formed by reaction of tyrosine residues with peroxynitrite, indicating that both reactive nitrogen and oxygen species have been present). Nitrotyrosine staining is widely used to indicate

oxidative stress (39, 40). As seen in Figures 5A–5D, the COPD samples had greatly increased nitrotyrosine staining compared with control samples, indicating that the COPD lungs had been exposed to much higher levels of oxidative stress than had the control lungs. We also stained control versus advanced COPD slides for 8-hydroxyguanosine residues (8-HG; formed by reaction of ROS with the DNA base guanine) and found similar results (not shown).

Reaction of Recombinant NEP and Lung Homogenates with H_2O_2

H_2O_2 , a powerful oxidizer, was used to determine whether oxidation by ROS inactivates or decreases NEP activity. It is important to note that we used H_2O_2 only as a readily available, easy-to-use reagent that would model the response of NEP to a number of oxidants. As shown in Figure 6A, 24-hour incubation of recombinant human NEP with various concentrations of H_2O_2 leads to potent inactivation of residual rNEP activity (68% inhibition with 100 μ M H_2O_2 , more inhibition with higher concentrations), comparable to the results of Shinall and colleagues (19). Moreover, as shown in Figure 6B, NEP

Figure 3. Neprilysin (NEP) protein and mRNA expression are reduced in lung lysates from patients with chronic obstructive pulmonary disease (COPD) and pulmonary vascular remodeling. (A) NEP expression was determined by Western blot using NEP-specific antibody, $n = 13$ – 14 per group. $*P < 0.017$ compared with control (0.05/3, upon application of Bonferroni correction for multiple comparisons). (B, upper panel) Representative Western analysis of control and COPD lung homogenates (30 μ g each), probed with anti-human NEP antibody as described in the METHODS. The two lanes were not adjacent to one another on gel, but were from the same gel and were treated identically. (B, lower panel) Representative nitrocellulose membrane stained with Coomassie Blue, after transfer of human lung homogenates, routinely used as a loading control, as well as a check on sample and transfer integrity. (C) NEP mRNA expression was determined by quantitative real-time polymerase chain reaction using human NEP primers from Qiagen. $n = 9$ per group.

Figure 4. Neprilysin (NEP) expression is decreased in alveolar walls and distal (25–100 μ m) remodeled vessels in chronic obstructive pulmonary disease (COPD) lungs compared with control lungs. (A) Human lung sample slides were stained with anti-NEP antibody at 1:100 dilution and blindly reviewed. The intensity of NEP signal in alveolar walls and in the distal pulmonary vessels (25–100 μ m) in each sample were scored from 0 to 4+. Nine areas were analyzed from each lung sample and the score was averaged for each lung. Open bars, control samples; solid bars, COPD samples. $n = 5$ lung samples for control or COPD. * $P < 0.001$ compared with control alveolar walls; ** $P < 0.001$ compared with all other groups. Control alveolar walls did not differ significantly from control distal vessels. (B and C), Representative images of NEP-stained distal vessels from control and COPD samples. (B) Control lung, $n = 6$. (C) Advanced COPD lung with pulmonary vascular remodeling, $n = 9$. NEP signal is brown (DAB). Arrows point to endothelial cells. Arrowheads point to alveolar wall. Scale bars = 50 μ m.

activity present in control crude lung homogenates is also inactivated by H_2O_2 (38% inhibition with 100 μ M H_2O_2 after 24 h).

NEP Activity and Expression in Human PA SMCs

To begin to understand some of the mechanisms responsible for the decreases in NEP activity/expression that occur in COPD lung, and to follow up on our observation that NEP expression is decreased in the distal remodeled vasculature, we moved to a simplified cell system using human PA SMCs. Because SMC proliferative changes have been observed at both proximal and distal sites in the pulmonary vasculature (41) and proximal-derived human PA SMCs are much easier to obtain, we used proximal PA SMCs to complete these initial studies.

As exposure to CSE, hypoxia, and ROS may mimic some of the conditions leading to COPD and vascular remodeling *in vivo*, human PA SMCs were exposed to normoxia (20% O_2), 5 μ g/ml CSE, hypoxia (3% O_2), or 100 μ M H_2O_2 for 48 hours, and the patterns of NEP activity and protein and mRNA expression were compared with those of control versus COPD lungs. Shown in Figure 7A is a representative light image of human PA SMCs with characteristic spindle shape. As shown in Figures 7B–7D, PA SMC NEP activity was decreased by 30 to 39% (Figure 7B), protein expression was decreased by 28 to 38% (Figure 7C), and relative mRNA levels were decreased by 11 to 48% (Figure 7D), compared with the normoxic control, after 48-hour exposure to CSE, hypoxia, or H_2O_2 . Note that, *in vitro*, NEP activity, protein expression, and mRNA expression were all decreased to about the same extents, in contrast to

Figure 5. Nitrotyrosine staining for oxidant-damaged proteins is higher in chronic obstructive pulmonary disease (COPD) lungs compared with control lungs. The presence of nitrotyrosine residues on proteins can be used as a marker for peroxynitrite formation and indicates oxidant damage to proteins. (A, C) Control lung, $n = 2$. (B, D) Advanced COPD lung, $n = 3$. Shown are images of (A, B) lung parenchyma or (C, D) distal (25–100 μ m) pulmonary vessels. Nitrotyrosine signal is brown (DAB). Black scale bars = 50 μ m; striped scale bars = 100 μ m.

Figure 6. Neprilysin (NEP) activity is susceptible to oxidation by H_2O_2 . (A) Human recombinant NEP was incubated at 37°C for 24 hours with the indicated concentrations of H_2O_2 , diluted 40-fold, and assayed for residual NEP activity, $n = 1$. (B) Control whole human lung homogenates ($n = 6$, four different samples) were incubated at 37°C for 24 hours with or without 100 μM H_2O_2 , diluted 60-fold, and assayed for residual NEP activity.

what was observed *in vivo*. Also, H_2O_2 did not significantly decrease NEP mRNA expression (Figure 7D). However, in preliminary experiments, we have observed a transient but significant decrease (40%) in NEP mRNA after 4-hour exposure to H_2O_2 (not shown).

Prevention of Decreases in NEP Activity by Various Antioxidants

If oxidant stress is an important mechanism for decreasing NEP activity *in vivo*, then an antioxidant should be able to prevent at least some of the observed decreases in PA SMC NEP activity. Therefore, human PA SMCs were incubated for 0.5 hour with the antioxidant tiron (2.5 mM) and exposed to normoxia, CSE,

hypoxia, or H_2O_2 for 4 hours, which is sufficient time to observe NEP activity, but not protein expression, losses. As demonstrated in Figure 8, without tiron, NEP activity was significantly decreased after 4-hour exposure to CSE, hypoxia, and especially H_2O_2 . All decreases in NEP activity were prevented by the antioxidant tiron. An additional experiment was performed in the same manner with the antioxidants tiron, MnTMPyp (a dual SOD/catalase mimetic) (34), and PEG-conjugated catalase, to address how tiron, which is known for its SOD mimetic properties, may reverse the effects of H_2O_2 on NEP activity. All three antioxidants (MnTMPyp, PEG-catalase, and tiron) prevented H_2O_2 -induced decreases in PA SMC NEP activity (not shown).

Figure 7. Cigarette smoke extract (CSE), hypoxia (Hx), and a potent reactive oxygen species (H_2O_2) decrease neprilysin (NEP) activity and expression in human pulmonary arterial (PA) smooth muscle cells (SMC). (A) Light microscopic image of normoxic human PA SMC (Clo-netics). Note characteristic spindle shape. (B–D), PA SMC were exposed to normoxia (Nx; 20% O_2), CSE (5 $\mu g/ml$), hypoxia (3% O_2), or H_2O_2 (100 μM), as indicated, for 48 hours. Values for normoxic control cells (1.00) were used to normalize others. (B) NEP catalytic activity, $n = 4$ –8 (four to six cell populations). (C) NEP protein expression, measured by Western analysis and calculated by densitometry, $n = 5$ (four cell populations). (D) NEP mRNA levels measured by quantitative real-time polymerase chain reaction, $n = 3$ (three cell populations). * $P < 0.05$ versus normoxia (Nx).

Figure 8. Prevention of decrease in neprilysin (NEP) activity by the antioxidant tiron. Human pulmonary arterial (PA) smooth muscle cells (SMCs) were incubated with 2.5 mM of the antioxidant tiron 0.5 hour before and throughout 4-hour exposure to normoxia (Nx; 20% O₂), cigarette smoke extract (CSE) (5 µg/ml), hypoxia (3% O₂; Hx), or H₂O₂ (100 µM). NEP catalytic activity was then determined fluorometrically. Four bars without tiron: Nx, CSE, Hx, n = 8. H₂O₂, n = 7. Four bars with tiron: Nx, CSE, Hx, n = 4. H₂O₂, n = 3, *P < 0.05 versus Nx alone. Nx with tiron did not differ significantly from Nx alone, nor from CSE, Hx, or H₂O₂ (also with tiron).

Prevention of NEP Protein Degradation by Lysosomal and Proteasomal Inhibitors

Because COPD lung NEP protein expression is decreased more than is NEP mRNA expression (48 vs. 30%; Figures 3A and 3C), a mechanism in COPD lung which may be involved in this differential effect is an increase in NEP protein degradation. Because NEP is a membrane protein, we considered both lysosomal and proteasomal protein degradation mechanisms. After serum withdrawal, human PA SMCs were preincubated for 2 hours with the lysosomal protein degradation inhibitor folimycin (50 nM) and exposed to normoxia, CSE, hypoxia, or H₂O₂ for 48 hours. Cell lysates were then examined for NEP protein expression by Western analysis. Although folimycin did not have much effect on NEP protein expression under normoxic conditions, it provided nearly complete protection to the NEP protein from CSE or hypoxia exposure, whereas protection from H₂O₂ exposure was somewhat weaker (10–40%; not shown). Similar experiments conducted with the proteasomal protein degradation inhibitor Clasto-lactacystin β-lactone (1 µM) indicated that this inhibitor provided only partial protection to the NEP protein (20–65%) from all three types of exposures (CSE, hypoxia, or H₂O₂; not shown).

DISCUSSION

The current study demonstrates that NEP is likely an important factor in the regulation of susceptibility of humans to pulmonary vascular remodeling in response to smoke inhalation and hypoxia. We previously demonstrated that loss of NEP increases susceptibility to pulmonary vascular remodeling and PHTN in chronically hypoxic mice (17). The present work was undertaken because of the importance of extending findings made in animal models to human tissue. In COPD, pulmonary vascular remodeling requires several factors, including cigarette smoke, oxidant stress, inflammation, and intermittent or persistent hypoxia. Hypoxia may play a particularly important role in the PHTN of at least a portion of patients with COPD (8). A number of additional factors and pathways, including IL-6 and

5-HTT (also 5-HT itself) and bone morphogenic protein, may contribute to COPD-associated PHTN and variable susceptibility to PHTN in COPD (6, 10, 13, 14, 42). Our work suggests that NEP may also contribute to variable susceptibility to COPD-associated PHTN. Likewise, there is a wide range of expression levels of NEP in human lung (43). Furthermore, our immunostaining results suggest that NEP protein expression in COPD lung, and also in lungs of humans with other causes of PHTN, is decreased more in the distal vasculature, where prominent remodeling is observed. This may be taken as additional evidence of the importance of NEP in pulmonary vascular disease. Whereas NEP expression in alveolar walls of COPD lung is low, this does not conflict with our finding that NEP expression is even lower in the distal pulmonary vasculature of COPD lung. NEP expressed on alveolar wall cells regulates the local neuropeptide balance and microenvironment and thus impacts nearby vascular cells.

NEP is involved in several signaling pathways, by both peptidase-dependent and -independent mechanisms (44, 45); it is not clear which of these pathways may influence PA SMC biology. We postulate that the loss of NEP activity/expression leads to the proliferation/migration of dedifferentiated SMCs or myofibroblasts into the distal circulation, promoting pulmonary vascular remodeling and PHTN. However, besides proliferative and migratory effects, the loss of NEP may lead to vasoconstrictive, angiogenic, and inflammatory effects on many cell types, particularly on the PA SMCs of the distal pulmonary vasculature. Our laboratory has found (17, 46) that serum, select neuropeptides, and growth factors such as platelet-derived growth factor (PDGF) have proliferative and migratory effects on mouse PA SMCs, which are inversely dependent on NEP (i.e., these effects are increased or decreased when NEP is decreased or increased, respectively). NEP-null PA SMCs also have increased proliferative responses to hypoxia in the presence of trace serum and neuropeptides (17). However, the magnitude of the responses to hypoxia, CSE, or H₂O₂ are quite small compared with those of major peptide mitogens, such as PDGF alone (not shown). Recently, we obtained evidence that one mechanism whereby NEP inhibits the proliferation and migration of wild-type mouse PA SMCs is through inhibition of the association of Src-kinase and the PDGF receptor (46).

The NEP substrates endothelin-1 (47) and the bombesin-like peptides (48) have proliferative and vasoconstrictive properties; basic fibroblast growth factor-2 (FGF-2; 155 amino acids) has potent angiogenic effects (45). Decreases in NEP would be expected to increase the effects of these NEP substrates. The antiinflammatory effects of NEP (49) are important also, as it is believed that inflammation contributes to pulmonary vascular remodeling in COPD (9). Proinflammatory peptide substrates, such as substance P and bradykinin, are degraded by NEP. NEP has also been found to reduce local concentrations of select proinflammatory mediators, likely through peptidase-independent interactions with other signaling molecules (50).

NEP may be involved in pathways with other mediators that have been proposed to play roles in PHTN or COPD-associated PHTN, such as 5-HT (or 5-HTT). NEP gene deletion is associated with increased numbers of pulmonary neuroendocrine cells (17), which secrete, among other neuropeptides and amines, the pulmonary vasoconstrictor 5-HT (51). Because patients with COPD may have up to twofold increases in the number of pulmonary neuroendocrine cells compared with normal smokers (52), NEP may be negatively correlated with pulmonary hypertensive levels of 5-HT. We plan to investigate interactions between NEP and other possible mediators of COPD-associated PHTN, such as 5-HT (or 5-HTT), IL-6, or bone morphogenic protein, in future studies. We believe that

the above facts and findings strongly suggest an inverse functional link between NEP and pulmonary vascular remodeling.

All of our advanced COPD lung samples showed evidence of pulmonary vascular remodeling (including a dropout of the distal vessels and vascular wall thickening). Although we cannot obtain direct PA pressure data on the majority of these patients, the vascular remodeling data are sufficient to indicate the likely presence of PHTN either at rest or with exercise. We found in our advanced COPD samples a dropout, or decreased density, of the small (25–100 μm) pulmonary vessels (Figure 1A). Matsuoka and colleagues (53) recently demonstrated that, among patients with severe COPD, there is a significant correlation of rarification or dropout of the small, distal pulmonary vessels with PA pressure, measured at rest. This relationship indicates that many of our patients with COPD may have had rest PHTN. Second, as Kubo and coworkers have shown (10), pulmonary vascular wall thickness in patients with advanced COPD is significantly related to exercise-induced PHTN. We conclude that a large proportion of our patients with advanced COPD had rest PHTN, but in addition, most, if not all, of our patients would have had PHTN induced by exercise.

CS-induced damage to endothelial cells is considered to be the primary alteration that initiates PHTN in COPD (4). Chronic CS exposure causes a thickening of the endothelial (intimal) layer of the pulmonary vessels, where SMC-like cells have been found. Although these intimal changes contribute to the remodeling found with COPD, medial and adventitial changes become more prominent in advanced disease (54), as we have found in our patients with advanced COPD. It is unknown what causes the progression from CS injury to PHTN associated with COPD. We propose that a decrease in lung NEP activity/expression may be one of these factors.

In lung tissue, we found a differential effect, that is, NEP activity in COPD (vs. control) lungs was decreased by 76%, whereas NEP protein expression in COPD lungs was only decreased by 48%. This differential effect may at least in part involve ROS-inactivation of the NEP enzyme through chemical adduct formation, while still allowing antibody detection of the NEP protein (19, 55). ROS present in COPD lungs may come directly from cigarette smoke products, or may be generated in COPD lungs due to inflammatory responses or localized hypoxia (40, 56, 57). We found that COPD lung tissue has much higher levels of nitrotyrosine and 8-HG residues when compared with controls (indicating greater oxidative stress in the COPD samples), that NEP activity is directly inactivated by H_2O_2 (which models the response of NEP to a number of oxidants in general), and that an antioxidant (tiron) prevents loss of NEP activity due to CSE, hypoxia, or H_2O_2 , further strengthening this connection between reduced NEP activity and ROS in COPD. Although tiron acts as an SOD mimetic, it also is able to chelate certain metal ions, and has been shown to react with (“scavenge”) hydroxyl radicals (which are likely the major reactive species through which H_2O_2 acts) at 100 times the rate that it reacts with superoxide radicals; tiron has been characterized as an electron trap (58). A preliminary experiment with other antioxidants with catalase-like activity suggests that, like these other antioxidants, in our experimental systems, tiron is able to combat oxidation by H_2O_2 .

We also found differential decreases of NEP protein and mRNA levels (48 and 30% decreases in control vs. COPD, respectively), which may involve inductions of lysosomal and/or proteasomal NEP protein degradation in COPD lung. Because NEP is associated with the cell membrane, we presumed that a substantial amount of NEP protein may be degraded in the lysosome; however, proteasomal mechanisms could not be ruled

out. We found evidence that both types of mechanisms may be of importance in mediating NEP protein degradation in PA SMCs. However, Malhotra and colleagues have found that proteasomal expression is decreased in humans with severe COPD (59). This may mean that lysosomal, rather than proteasomal, NEP protein degradation is more important in humans with COPD.

We have not investigated mechanisms resulting in decreases in NEP mRNA. In COPD, there may be important changes in the concentrations of or interactions of NEP with various transcription factors, or of NEP gene methylation. These mechanisms will be addressed in future work.

We acknowledge the current studies have limitations, but we do not think these issues in any way compromise our conclusions. Human lung samples are difficult to obtain, as they are often collected during major invasive procedures. If they are obtained from tissue donors, they may be accompanied by concerns about ischemic time during the harvest. Lung tissue may be subject to a satellite effect from adjacent tumors or granulomas. Unintentional variations in harvest and preparation of the samples make morphometric analyses more technically difficult, but these variables did not impact our activity and expression measurements. Morphometric analyses were also complicated by the heterogeneity observed in COPD lung sections (i.e., localized areas of advanced disease often coexist within or near areas of relatively normal-looking tissue). Our *in vitro* cell system, human PA SMCs exposed to CSE, hypoxia, and H_2O_2 , obviously represents an oversimplification, which did not model differential effects on NEP activity and expression observed *in vivo*. However, most patients with COPD are subject to varying conditions for years, whereas our treatments of the PA SMCs are short term, and they can be expected to only partially model some of the conditions, cell types, and tissue destructive factors present in COPD lungs. Proximal PA SMCs from humans were used as the best available model of distal PA SMCs (41), because it would have been very difficult, if not impossible, to obtain sufficient numbers of distal human PA SMCs for the current study. However, the pattern of SMC responses was consistent with observations made with lung tissue.

We have found that CS (not shown), like hypoxia (17), decreases NEP activity and expression in wild-type mouse lung. No adaptive changes in other relevant lung peptidases, including DPPIV, endothelin-converting enzyme, and angiotensin-converting enzyme, were found in mouse lung when NEP levels were reduced (17). In human lung, we have also observed by immunohistochemistry that CS inhibits NEP protein expression (not shown), but our present results indicate that CS may not affect NEP mRNA much. The decreases observed here in humans in NEP activity/expression are not due to general cell loss and may also be selective for NEP. We compared decreases observed for NEP in human lung to another peptidase, DPPIV, which in many respects may be similar to NEP but has important differences (23, 24, 26). Both NEP and DPPIV are found within the lung, as well as within many other tissues, are cell surface type II membrane peptidases, and have similar structures, despite the fact that DPPIV is a serine aminopeptidase, whereas NEP is a zinc endopeptidase. Both peptidases are involved in the degradation of substance P, bradykinin, neuropeptide Y, and to a varying extent, VIP (20–24), but both enzymes have other substrates that are unique to each. In addition, DPPIV is much more actively involved in the degradation of VIP than is NEP. NEP can hydrolyze VIP when it reaches high concentrations, resulting in the generation of several peptides that are still active (22). Also, in contrast to NEP, DPPIV inhibition tends to promote lung function (26). DPPIV activity/protein expression did not vary between control and COPD

lungs. Our studies do not exclude the possibility that other lung peptidases, such as angiotensin-converting enzyme 2 (60), may also contribute to the pathogenesis of PHTN in chronic diseases such as COPD. In future work, we plan a wider analysis of these and other peptidases in control and diseased tissue.

Because of the high incidence of COPD and the increased availability of characterized lung tissue, COPD is a useful human model for the study of mechanisms of pulmonary vascular disease induced by smoke inhalation and hypoxia. There is much individual variation in the severity of COPD-associated PHTN and also in the normal range of NEP pulmonary activity/expression. Based on our results, we hypothesize that individuals may vary in their susceptibility to pulmonary vascular remodeling and PHTN depending on their level of lung NEP activity/expression. Our studies could lead to new treatments based on the concept that maintaining or increasing lung NEP may protect against PHTN in response to chronic smoke and hypoxia.

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