Hypoxia decreases lung nephrilysin expression and increases pulmonary vascular leak

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Received 18 April 2001; accepted in final form 25 May 2001

Carpenter, Todd C., and Kurt R. Stenmark. Hypoxia decreases lung nephrilysin expression and increases pulmonary vascular leak. Am J Physiol Lung Cell Mol Physiol 281: L941–L948, 2001.—Although prior studies suggest that hypoxia may increase pulmonary vascular permeability, the mechanisms responsible for that effect remain uncertain. Neprilysin (neutral endopeptidase) is a cell surface metallopeptidase that degrades several vasoactive peptides including substance P and bradykinin. We hypothesized that hypoxia could reduce lung nephrilysin expression, leading to increased vascular leak. Weanling rats were exposed to normobaric hypoxia (inspired O2 fraction = 0.1). Lung nephrilysin activity was significantly decreased after 24 and 48 h of hypoxia (P < 0.006). The decrease in enzyme activity was associated with decreased lung nephrilysin protein content and decreased lung nephrilysin mRNA expression. Immunohistochemistry showed a predominant perivascular distribution of nephrilysin, with clear reductions in nephrilysin immunoreactivity after exposure to hypoxia. Exposure to hypoxia for 24 h also caused marked increases in vascular leak (P = 0.008), which were reversed by the administration of recombinant nephrilysin. The hypoxia-induced increase in leak was also reversed by substance P and bradykinin receptor antagonists. We conclude that in young rats hypoxia decreases lung nephrilysin expression, which contributes to increased pulmonary vascular leak via substance P and bradykinin receptors.

The effects of acute exposure to moderate hypoxia on lung fluid balance remain a subject of ongoing debate. Although pulmonary edema associated with high-altitude exposure is well documented in humans and is clearly associated with pulmonary arterial hypertension, the relative contributions of increases in vascular pressure and vascular permeability to that illness remain unclear. A significant body of literature suggests that hypoxia in and of itself can alter vascular permeability. Exposure to moderate environmental hypoxia has been shown to increase the extravasation of labeled albumin from the vascular bed into the lung in both adult and weanling rats, an effect augmented by inflammatory stimuli such as endotoxin or recent viral infection, although the mechanisms responsible for that increase in vascular leak induced by hypoxia are uncertain (3, 17, 19). In vitro exposure to more severe levels of hypoxia has also been shown to increase the permeability of endothelial cell monolayers in culture, apparently via mechanisms involving the release of interleukin-6 and oxygen radicals (1), although the relevance of those findings to in vivo exposure to more moderate levels of hypoxia remains unclear.

Neprilysin [neutral endopeptidase (NEP)] is a cell surface zinc metallopeptidase known to degrade a variety of vasoactive mediators. NEP is widely expressed in the lung in both humans and rodents, and several of the known substrates for NEP, including substance P and bradykinin, are also known to increase vascular permeability in experimental animals (10, 15). Interestingly, a recent study (15) has found diffuse increases in vascular permeability in mice with a targeted deletion of the NEP gene. In addition, Carpenter et al. (2) have previously reported preliminary results suggesting that exposure to moderate hypoxia can lead to a decrease in lung NEP expression in weanling rats. We hypothesized, then, that increases in lung vascular leak seen with exposure to hypoxia might be mediated by decreases in lung NEP expression.

To test this hypothesis, nephrilysin protein and mRNA expression were measured in the lungs of young rats exposed to moderate hypoxia. Vascular leak was assessed in normoxic and hypoxic rats by measuring Evans blue dye-labeled albumin escape, and the role of nephrilysin in controlling vascular leak was assessed by measuring albumin escape in rats given recombinant NEP or blockers of bradykinin and substance P receptors.

METHODS

Animals. Experimental animals were pathogen-free weanling male Sprague-Dawley rats purchased from a commercial vendor (Harlan Sprague Dawley, Indianapolis, IN). The animals arrived in Denver at age 20–22 days and weighing 50–60 g each and were allowed to acclimate for 7 days before hypoxic exposure. All animals were allowed free access to food and water and were subjected to a similar day-night light cycle. Animals were housed at Denver altitude (1,600 m) at all times.

Exposure to hypoxia. Animals in the hypoxic test groups were weighed and then exposed to normobaric hypoxia (in-
spired O₂ fraction (0.1) for periods of time up to 72 h. Immediately after hypoxic exposure, the animals were anesthetized with 80 mg/kg of pentobarbital sodium given intraperitoneally. A midline thoracotomy was then performed, and the animal was exsanguinated via cardiac puncture. The lungs and main stem bronchi to the carina were excised and weighed. The left lung was then removed, weighed separately, and dried in an oven at 55°C until its weight was stable for 24 h. The right lung was trimmed of the extrapulmonary airways, snap-frozen in liquid nitrogen, and stored at −70°C until processed for assay of NEP activity, protein, and mRNA levels. The calculations made were total lung weight-to-prehypoxia body weight ratio, expressed as milligrams of lung per gram of body weight, and wet-to-dry lung weight ratio. In addition, from animals in the normoxic control and 48-h hypoxic groups, the right kidney was excised, snap-frozen in liquid nitrogen, and stored at −70°C until processed for assay of NEP protein and mRNA levels.

To assess the effects of nephrilysin inhibition on lung water, a separate group of animals was given a single oral dose (50 mg/kg) of potentiobarbital sodium given intraperitoneally. A midline thoracotomy was then performed, and the animal was exsanguinated via cardiac puncture. The lungs and main stem bronchi to the carina were excised and weighed. The left lung was then removed, weighed separately, and dried in an oven at 55°C until its weight was stable for 24 h. The right lung was trimmed of the extrapulmonary airways, snap-frozen in liquid nitrogen, and stored at −70°C until processed for assay of NEP activity in the lung were due to changes in NEP protein content, lung homogenates were studied by standard Western blotting techniques. Briefly, the lungs from additional animals exposed to hypoxia for 24 or 48 h and from normoxic control animals were fixed by tracheal perfusion with phosphate-buffered 10% formalin at 25 cm H₂O pressure for 24 h. After formalin perfusion, the lungs were paraffin embedded and sectioned for immunostaining. Tissue sections were rehydrated through graded ethanol washes, and antigen retrieval was accomplished by heat treatment for 10 min in 10 mM sodium citrate, pH 6.0. The primary antibody used, at a 1:50 dilution, was the same monoclonal antibody as used for Western blotting. As a negative control, a nonspecific monoclonal mouse IgG1 primary antibody (Labvision) was used at a 1:50 dilution. Slides were developed with a commercially available alkaline phosphatase kit and substrate (VECTASTAIN kit and Vector Red substrate, Vector Laboratories, Temecula, CA) and were counterstained with hematoxylin, rehydrated, and permanently mounted.

RT-PCR studies. The expression of nephrilysin mRNA was assessed by relative RT-PCR. Total lung RNA was extracted with standard techniques. Briefly, the frozen lung tissue was homogenized in TRIzol (Sigma), and RNA was isolated following the manufacturer’s instructions. Isolated total lung RNA was then quantified by measurement in an ultraviolet (UV) spectrophotometer. RT to cDNA was accomplished by priming 5 μg total RNA/sample with oligo(dT) and then using the Superscript II reverse transcriptase kit (GIBCO BRL, Life Technologies, Gaithersburg, MD) to make cDNA followed by digestion with RNase H to remove untranscribed RNA. The cDNA preparations were diluted 1:10 in nuclease-free water, and PCRs were performed on 2 μl of diluted cDNA from each sample.

Primers were chosen for nephrilysin PCR based on the published coding sequence of the rat nephrilysin gene (GenBank accession no. M15944). The sequences of the primers used were 5′-GGCAACCTCTGCTCACACTGTTAC-3′ (bp 539–562) and 5′-GCATTGGGTTCATTTGTCTTC-3′ (bp 970–949). The identity of the 432-bp PCR product generated with these primers was confirmed by restriction analysis and partial sequence analysis. Nephrilysin primers were used at a final concentration of 400 nM. Control reactions with increasing cycle numbers confirmed that the reaction for the NEP product was in the exponential phase up to 30 cycles (data not shown), and all further studies were then done at 28 cycles. The PCR cycle used was 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s.

As an internal control for each PCR, RT-PCR amplification of β-actin mRNA was also done on each sample. Primers chosen for β-actin PCR were based on the published coding sequence of the mouse β-actin gene (GenBank accession no. M12481). The sequences of the primers used were 5′-CATC-CTGAAAAACCTCCTTATGCAAAC-3′ (bp 786–810) and 5′-CAAAGAAAGGGTGTAAACGCCGC-3′ (bp 1089–1066). The 304-bp PCR product generated with these primers spans intron 3 of the rat β-actin gene, and the identity of the PCR

L942 HYPOXIA DECREASES LUNG NEPRILYSIN

\[ \text{As an alternate means of assessing changes in NEP protein in the lung and to study the specific sites of those changes, formalin-fixed lung tissue was stained for NEP with standard immunohistochemistry techniques. Briefly, the lungs from additional animals exposed to hypoxia for 24 or 48 h and from normoxic control animals were fixed by tracheal perfusion with phosphate-buffered 10% formalin at 25 cm H₂O pressure for 24 h. After formalin perfusion, the lungs were paraffin embedded and sectioned for immunostaining. Tissue sections were rehydrated through graded ethanol washes, and antigen retrieval was accomplished by heat treatment for 10 min in 10 mM sodium citrate, pH 6.0. The primary antibody used, at a 1:50 dilution, was the same monoclonal antibody as used for Western blotting. As a negative control, a nonspecific monoclonal mouse IgG1 primary antibody (Labvision) was used at a 1:50 dilution. Slides were developed with a commercially available alkaline phosphatase kit and substrate (VECTASTAIN kit and Vector Red substrate, Vector Laboratories, Temecula, CA) and were counterstained with hematoxylin, rehydrated, and permanently mounted.} \]

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product was confirmed by restriction analysis. Actin primers were also used at a final concentration of 200 nM. Control reactions with increasing cycle numbers confirmed that the reaction for the β-actin product was in the exponential phase up to 20 cycles (data not shown), and all further studies were then done at 18 cycles. The PCR cycle used was 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s. Contamination of the RNA samples with genomic DNA was excluded by the absence of products from PCRs performed with RNA without RT as well as by the absence of higher molecular weight bands, indicating amplification of β-actin genomic DNA (data not shown).

PCR products were loaded onto a 1.5% agarose-Tris-ace
tate-EDTA gel, separated by electrophoresis, and visualized with ethidium bromide. Gels were photographed under UV illumination, and the images were scanned into a computer for densitometric analysis. Final results are expressed as the ratio of neprilysin PCR product to β-actin PCR product for each sample. All experiments were repeated three times with similar results.

Northern blotting studies. To confirm the results of our RT-PCR experiments, the expression of rat neprilysin mRNA was also assessed by Northern blotting. Total lung RNA was isolated and quantified as described in RT-PCR studies. Northern blotting was performed with a commercial kit following the manufacturer's instructions (Northern-Max Glyoxal, Ambion, Austin, TX). Briefly, 10 μg RNA/sample were glyoxal treated, separated by electrophoresis in a 1% agarose gel, and transferred to positively charged nylon membranes (Hybond-N+, Amersham Pharmacia) by downward alkaline transfer. Once transferred, the blots were UV cross-linked to fix the RNA to the membrane. Detection was accomplished with digoxigenin-labeled RNA probes. The blots were prehybridized for 30 min at 68°C in hybridization solution (UltraHyb, Ambion), and then the probe was added at a final concentration of 10 ng/ml and hybridized to the blot overnight at 68°C. After low- and high-stringency washes, the hybridized probe was detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Diagnostics, Indianapolis, IN) and CSPD chemiluminescent sub
cstrate.

The probes for rat neprilysin were constructed with the primers listed in RT-PCR studies. The PCR products from these primers were ligated into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA), and antisense RNA probes were produced by in vitro transcription with a digoxigenin-labeling kit (Roche Diagnostics). As a control for RNA loading and transfer, the blots were stripped and reprobed with a digoxigenin-labeled antisense 18S rRNA probe transcribed from a commercially available template (Ambion). Results are expressed as the ratio of neprilysin signal mRNA to 18S RNA for each sample. Northern blotting experiments were performed twice each with similar results.

Measurement of vascular protein leak. The leakage of pro
tein from the pulmonary vasculature in control and hypoxic animals was assessed by measuring the accumulation of Evans blue dye in the lung. After exposure to room air or to 24 h of hypoxia, animals were anesthetized with ketamine (80 mg/kg ip) and xylazine (20 mg/kg ip) and then injected via a tail vein with 30 mg/kg of Evans blue dye. After Evans blue dye injection, control animals remained in room air for 10 min, and the hypoxic animals were returned to hypoxia for 10 min. After that time, the heart-lung block was excised, the lungs were perfused free of blood with phosphate-buffered saline via the pulmonary artery, and then the extrapulmo
nary airways and tissues were removed. The right lung was weighed and then incubated in formamide for 18 h at 37°C to extract the Evans blue dye. The left lung was weighed and then dried in an oven at 55°C, and the wet-to-dry weight ratio from that lung was used to estimate the dry weight of the right lung. The extracted dye was quantitated in a spectrophotometer by measuring absorbance at 620 nm with standards of Evans blue dye dissolved in formamide, and Evans blue dye extravasation is expressed as nanograms of Evans blue dye per milligram of dry tissue.

To verify that the increase in leak with hypoxia was due to the decrease in NEP expression, normoxic and hypoxic animals were injected with 3 mg/kg of recombinant human NEP (Axys Pharmaceuticals, South San Francisco, CA) and then had Evans blue dye extravasation measured as above. To begin to investigate the identity of the biochemical mediators responsible for the increase in leak, normoxic and hypoxic animals were injected with 1 mg/kg of SR-140333 (a gift from Dr. X. Emonds-Alt, Sanofi-Recherche, Montpellier, France), a neurokinin-1 (NK1) receptor antagonist or with 1 mg/kg of HOE-140 (American Peptide, Sunnyvale, CA), a bradyk
in-2 (B2) receptor antagonist. The receptor blockers and recombinant enzyme were injected 10 min before administration of Evans blue dye as described above.

Statistical analysis. All results are expressed as means ± SE unless otherwise noted. Comparisons between groups were analyzed with Student's t-test for two-group comparisons or one-way analysis of variance with Fisher's protected least squares distribution posttest for multiple comparisons. Correlations were measured by simple linear regression analysis. Statistical analyses were done with Statview 5.0 statistical analysis software (SAS Institute, Durham, NC) on an Apple Macintosh computer. Differences were considered significant when P < 0.05.

RESULTS

Hypoxia decreases lung neprilysin activity. To test the hypothesis that exposure to hypoxia decreases lung neprilysin activity, neprilysin enzyme activity was assayed on aliquots of lung homogenate from control animals (n = 7) and from animals exposed to normobaric hypoxia for 12 h (n = 6), 24 h (n = 6), 48 h (n = 6), or 72 h (n = 4). Exposure to hypoxia led to a progressive decrease in lung NEP activity with increasing duration of exposure to hypoxia, with those decreases first becoming significant at 24 h of hypoxia (P < 0.006; Fig. 1). This effect was greatest after 48 and 72 h of hypoxia when lung NEP activity was 57% of the value in control lungs.

Hypoxia decreases lung neprilysin protein expression. To determine whether the decreases in lung neprilysin activity associated with hypoxia were due to decreases in lung neprilysin protein expression, neprilysin protein content was measured in lung homogenates by Western blotting. As shown in Fig. 2, exposure to hypoxia for 24 and 48 h led to significant decreases in lung neprilysin protein content (control, 0.88 ± 0.11 arbitrary units; 24-h hypoxia, 0.54 ± 0.03 arbitrary units; 48-h hypoxia, 0.30 ± 0.04 arbitrary units; P = 0.0007; n = 4 animals for control and 24-h hypoxia and 5 animals for 48-h hypoxia).

As an alternate means of assessing lung neprilysin protein expression and to determine whether the changes in NEP expression were localized to a specific anatomic site within the lung, NEP was also studied by immunohistoch
metry. In lungs from normoxic con

AJP-Lung Cell Mol Physiol • VOL 281 • OCTOBER 2001 • www.ajplung.org
Hypoxia decreases renal NEP expression. To determine whether this effect of hypoxia on NEP expression was specific to the lung, NEP protein expression was measured by Western blotting with kidney homogenates from normoxic and 48-h hypoxic animals. Similar to the findings in the lung, exposure to hypoxia for 48 h led to a significant decrease in renal NEP protein expression (normoxic control, 1.7 ± 0.3 arbitrary units for NEP to β-actin; 48-h hypoxia, 0.7 ± 0.2 arbitrary units for NEP to β-actin; *P < 0.03; n = 4 animals/group). Exposure to hypoxia for 48 h also decreased renal NEP mRNA expression as assessed by Northern blotting by a similar proportion (normoxic control, 0.76 ± 0.06 arbitrary units for NEP to 18S; 48-h hypoxia, 0.42 ± 0.1 arbitrary units for NEP to 18S; *P = 0.04; n = 4 animals/group).

Effects of hypoxia on lung vascular leak. To determine whether hypoxia alone led to increases in lung vascular leak in young rats, Evans blue dye extravasation was measured in a group of animals (n = 6/group) exposed to room air or to hypoxia for 24 h. Animals exposed to hypoxia showed a significantly greater vascular leak than normoxic control animals (203.2 ± 16.7 vs. 100.7 ± 15.1 ng Evans blue dye/mg dry tissue; *P = 0.008).

To determine whether the increases in lung vascular leak seen with hypoxia were related to a decrease in lung NEP expression and activity, Evans blue dye extravasation was measured in normoxic (n = 5) and hypoxic (n = 7) animals given recombinant human neprilysin. Although the administration of recombinant NEP did not significantly alter the amount of Evans blue dye leak in normoxic rats, the exogenous enzyme blocked the increase in leak in hypoxic rats (*P = 0.01; Fig. 5).

Effects of B₂ and NK₁ receptor antagonism on lung vascular leak. To determine whether the increases in lung vascular leak seen with hypoxia are mediated via activation of the NK₁ receptor, Evans blue dye extravasation was measured in normoxic and hypoxic animals (n = 4/group) given the NK₁ receptor antagonist SR-140333. Although SR-140333 did not alter the degree of vascular leak in normoxic animals, it did block the increase in leak in hypoxic animals (*P = 0.006; Fig. 6).

To determine whether activation of the B₂ receptor contributes to the increases in lung vascular leak seen with hypoxia, Evans blue dye extravasation was measured in normoxic and hypoxic animals (n = 4/group) given the B₂ receptor antagonist HOE-140. Although bradykinin receptor blockade did not alter the degree of vascular leak in normoxic animals, it did block the increase in leak in hypoxic animals (*P = 0.007; Fig. 6).

Effects of hypoxia and NEP inhibition on lung water. To determine whether the changes in lung NEP expression and pulmonary vascular leak seen with hypoxia led to changes in lung fluid balance, gravimetric lung water was measured in animals exposed to hypoxia for up to 72 h. Hypoxia alone led to significant increases in the lung weight-to-body weight ratio that were progressive over the course of 72 h. These increases in lung water

**Fig. 1.** Exposure to hypoxia decreased lung neprilysin [neutral endopeptidase (NEP)] enzyme activity. h12, h24, h48, and h72, 12, 24, 48, and 72 h of hypoxia, respectively. *P < 0.006 vs. normoxic control.

**Fig. 2.** Western blot showing decreased NEP protein content in lung homogenates from animals exposed to hypoxia for indicated times. β-Actin bands confirm equal protein loading.
displayed a significant inverse correlation with lung NEP activity \( (r = 0.59; P < 0.001; \text{Fig. 7}) \).

To determine whether the decreases in NEP activity led directly to changes in lung water, rats were treated for 24 h with the NEP inhibitor Sch-42495. NEP inhibition for 24 h in normoxic rats did not cause any increase in the lung weight-to-body weight ratio (control, 8.09 ± 0.07 mg/g; Sch-42495 treated, 7.90 ± 0.18 mg/g; \( P = 0.27; n = 6 \) animals/group) or the lung wet-to-dry weight ratio (control, 4.85 ± 0.03; Sch-42495 treated, 4.86 ± 0.04; \( P = 0.74; n = 6 \) animals/group) even though treatment with Sch-42495 decreased lung homogenate NEP activity by 60% (3.45 ± 0.4 vs. 1.54 ± 0.4 units/mg protein; \( P = 0.02)\), an amount comparable to the effect of 48 h of hypoxia. In contrast, in rats exposed to hypoxia for 24 h, NEP inhibition with Sch-42495 did lead to a small but significant increase in the lung weight-to-body weight ratio (hypoxia, 8.14 ± 0.08; hypoxia plus Sch-42495 treated, 8.58 ± 0.15; \( P = 0.01; n = 8 \) animals/group) and in the lung wet-to-dry weight ratio (hypoxia, 4.78 ± 0.02; hypoxia plus Sch-42495 treated, 4.89 ± 0.03; \( P = 0.03)\).

**DISCUSSION**

The major finding of this study was that subacute exposure to moderate hypoxia leads to a significant decrease in lung nephrilysin expression and activity.
This decrease in expression occurs at the mRNA and protein levels and appears to last for at least 72 h. In addition, the decrease in lung neprilysin expression seen with hypoxia is associated with an increase in lung vascular protein leak and that increase in leak is reversible with recombinant neprilysin as well as with blockers of the NK1 and B2 receptors. These results show not only that hypoxia can alter lung neprilysin levels but also that such changes in lung neprilysin expression may affect lung vascular leak and lung water balance that are, at least in part, mediated by substance P and bradykinin.

The finding that brief exposures to moderate degrees of hypoxia can affect lung neprilysin expression is novel. We found that with exposure to hypoxia, lung NEP activity and mRNA content begin to decrease as early as 12 h into the hypoxic exposure and that enzyme activity remains significantly reduced for up to 72 h. Previous work examining the effect of hypoxia on lung neprilysin expression is limited to one preliminary report (2) noting a decrease in NEP activity after 24 h of hypoxia, and one prior study (12) showing that chronic intermittent hypoxia can decrease lung NEP activity. The present study is consistent with those earlier findings. Interestingly, we found that although NEP protein content as measured by Western blotting closely parallels the results obtained with measurement of enzyme activity, the mRNA levels in the lung appear to be recovering by 48 h of hypoxia. The effects...
of longer exposure to hypoxia on lung NEP expression, including whether the effect of hypoxia on NEP expression persists or whether NEP protein levels recover with acclimatization, remain to be studied. Although the present study did not address the mechanisms by which hypoxia might alter NEP expression, both our finding of a decrease in renal protein and mRNA content in these hypoxic animals and a prior study (16) showing that ischemia-reperfusion injury can decrease renal NEP expression suggest that these effects are not limited to the lung. Possible mechanisms by which hypoxia might exert this effect on NEP expression include the activation of transcription factors or the generation of other intermediary molecules such as reactive oxygen species or cytokines. The promoter region of the rat nephrilysin gene has been characterized and appears to include three distinct regulatory elements (14), although no study to date has described hypoxia-responsive elements in the promoter region of the nephrilysin gene. Reactive oxygen species and cytokines can also contribute to increases in vascular permeability under hypoxic conditions in vitro (1), but the effects of those mediators on NEP expression have not been well studied and some cytokines may increase NEP expression in lung fibroblasts (11).

We also found that hypoxia increased vascular protein extravasation in the lung, with Evans blue dye-labeled albumin extravasation doubling after 24 h of hypoxia. This result agrees with previous studies in both adult and weanling rats (3, 19) as well as with some studies (7, 8, 13) that have suggested that humans at altitude may experience a generalized increase in capillary permeability. Our finding that the administration of recombinant nephrilysin can almost totally block the increases in leak seen with hypoxia suggests strongly that a decrease in lung NEP plays a major role in mediating hypoxia-induced increases in lung vascular leak. In support of this idea, NEP has recently been implicated in the control of vascular permeability in mice because a study (15) in transgenic mice deficient in NEP found significant increases in microvascular leak in the bladder and small bowel mucosa compared with those in wild-type control animals. It is also important to note as a limitation of our data that although previous studies (15, 19) have shown that both hypoxia and NEP deficiency can lead to increases in vascular permeability, our results do not address the relative contributions of increases in permeability and increases in pressure to vascular leak in this setting nor do they provide precise information about the anatomic site of the leak seen with hypoxia.

In addition, a role for NEP in controlling lung vascular leak is supported by our immunostaining showing that NEP protein expression in the lung is predominantly localized to perivascular areas and alveolar septa. Reduced activity of this perivascular enzyme could then lead to exaggerated effects on the lung vasculature by local mediators released in the lung parenchyma and interstitium. These findings are consistent with an earlier work (9) showing that NEP immunoreactivity appears primarily in the alveolar septum in human fetal and adult lungs and with a study (6) showing that although all three layers of the wall of the aorta express NEP, the highest level of expression is in the adventitia. The precise mechanisms by which such perivascular release of mediators might alter endothelial protein leak remain unclear but could include alterations in endothelial permeability, increases in microvascular pressure, or both. Interestingly, those vessels demonstrating the most intense perivascular staining for NEP in the normoxic lung tended to be thin-walled and not accompanied by airways, suggesting that those vessels may be on the venous side of the lung circulation. Whether NEP is expressed differently in pulmonary arteries compared with pulmonary veins and whether the different segments of the circulation are affected differently by changes in NEP expression remain to be studied in greater detail.

As mentioned above, the effects of decreased NEP expression on lung vascular leak are most likely the result of reduced degradation of NEP substrates that are vasoactive mediators. Two such NEP substrates are substance P and bradykinin, both of which are known to increase permeability in some vascular beds as well as to have effects on vascular tone. Substance P acts to increase vascular leak through binding to its receptor, the NK1 receptor (4). By blocking the NK1 receptor with SR-140333, a highly selective and long-acting antagonist (5), we were able to nearly completely block the increase in vascular leak in animals exposed to hypoxia. Bradykinin likewise acts to increase vascular permeability by binding to the B2 receptor (18). By blocking the B2 receptor with the specific antagonist HOE-140, we were again able to nearly completely block the increase in vascular leak in animals exposed to hypoxia. These studies suggest that hypoxia increases vascular leak, at least in part, as a result of the effects of substance P and bradykinin in the lung. These results are again consistent with a recent study (15) in transgenic mice deficient for NEP, which found in similar fashion that the increases in permeability in those animals were mediated via the NK1 and the B2 receptors, suggesting that substance P and bradykinin were involved in the control of permeability in those animals. In addition, the finding that NK1 and B2 receptor blockade resulted in a similar and near-complete reversal of the effects of the hypoxia-associated vascular leak may suggest that substance P and bradykinin act to increase leak in this setting via the same mechanism. This result is not unexpected given that some of the vascular effects of bradykinin are thought to occur via stimulation of substance P release and that a similar result was noted in NEP-deficient mice (15).

Finally, we found evidence to suggest that lung NEP activity not only affects vascular protein extravasation but may also affect overall lung fluid balance. We found that in hypoxic animals, lung NEP activity correlated inversely with gravimetric lung water as measured by the lung weight-to-body weight ratio. In addition, NEP inhibition with the long-acting NEP antagonist Sch-
42495 led to augmented lung water accumulation in hypoxic animals. Interestingly, NEP inhibition for 24 h with Sch-42495 had no effect on gravimetric lung water in normoxic rats, suggesting that decreases in NEP alone are not sufficient to lead to the accumulation of lung water and that the development of early pulmonary edema requires the presence of additional physiological factors, perhaps such as increased pulmonary vascular pressures.

In summary, this study shows that the subacute exposure of young animals to moderate hypoxia leads to a marked decrease in lung nephrins expression. In addition, such exposure to hypoxia leads to significant increases in lung vascular leak that are reversible with the administration of exogenous nephrin or with antagonists of the receptors for two nephrin substrates known to increase vascular leak, substance P and bradykinin. These findings suggest that the effect of hypoxia in increasing lung vascular leak is mediated, at least in part, by the loss of lung nephrins activity and that such changes may lead to the accumulation of lung water. Further study is needed to ascertain the molecular mediators of this effect of hypoxia on nephrin expression as well as its clinical relevance.

T. C. Carpenter was supported by a Beginning Grant-in-Aid from the American Heart Association Desert-Mountain Affiliate and by National Heart, Lung, and Blood Institute (NHLBI) Mentored Clinician Scientist Development Award HL-04483. K. R. Stenmark was supported by NHLBI Specialized Center of Research Grant HL-47144 and NHLBI Program Project Grant HL-14983.

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