Endothelin receptor blockade decreases lung water in young rats exposed to viral infection and hypoxia

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Carpenter, Todd C., and Kurt R. Stenmark. Endothelin receptor blockade decreases lung water in young rats exposed to viral infection and hypoxia. Am J Physiol Lung Cell Mol Physiol 279: L547–L554, 2000.—Viral respiratory infections may increase the susceptibility of young animals to hypoxia-induced pulmonary edema. Because hypoxia stimulates endothelin production, we hypothesized that an increase in lung endothelin contributes to these alterations in lung water. Weanling rats were infected with Sendai virus, causing a mild respiratory infection. At day 7 after infection, animals were exposed to hypoxia (inspired \( O_2 \) fraction = 0.1) for 24 h. Exposure to virus plus hypoxia led to increases in lung water compared with control groups (\( P < 0.001 \)). Lung endothelin levels were significantly higher in the virus plus hypoxia group than in control groups (\( P < 0.001 \)). A second group of infected animals received bosentan, a nonselective endothelin receptor antagonist, during exposure to hypoxia. Bosentan-treated animals showed less lung water accumulation, less lung lavage fluid protein, and less perivascular fluid cuffing than untreated animals (\( P < 0.01 \)). We conclude that the combination of a recent viral respiratory infection and exposure to moderate hypoxia led to increases in endothelin in the lungs of young rats and that endothelin receptor blockade ameliorates the hypoxia-induced increases in lung water found in these animals.

high-altitude pulmonary edema; Sendai virus; bosentan; preproendothelin

HIGH-ALTITUDE PULMONARY EDEMA (HAPE) is a form of noncardiogenic pulmonary edema that develops on ascent to altitude in susceptible individuals. Despite much research into the mechanisms underlying this process, the biochemical mediators leading to the development of HAPE remain obscure. Prior studies have shown that HAPE is uniformly associated with elevated pulmonary artery pressures, and much of the research into the mechanisms underlying HAPE has focused on the pulmonary hemodynamic alterations associated with hypoxia. Whether inflammatory processes act as a trigger to HAPE or occur only as sequelae to the illness once established remains controversial. Prior work has shown, however, that hypoxia alone can alter pulmonary vascular permeability (21) and that inflammatory stimuli can augment that response (17). In addition, children diagnosed with HAPE often report having had a recent upper respiratory illness (5), and recent work from our laboratory has suggested that a recent viral respiratory infection can increase the likelihood of lung water accumulation in young animals exposed to moderate environmental hypoxia (1).

The endothelins are a group of small peptides with known vasoconstrictive effects as well as effects on lung inflammation and possibly on edema formation in the lung. Exposure to environmental hypoxia has been shown to increase plasma endothelin levels in both humans (14, 20) and experimental animals (6), and hypoxia increases the expression of the preproendothelin gene in rat lung (6). In addition, endothelin infusions led to pulmonary edema formation in isolated perfused lung models (9, 19). We hypothesized that the augmented lung water accumulation we previously reported in young animals exposed to the combination of a recent viral infection and environmental hypoxia might be mediated by elevated levels of endothelin in the lung.

To test this hypothesis, lung endothelin levels were measured in a series of animals exposed to a mild viral respiratory infection, moderate hypoxia, or both. In addition, to determine whether changes in lung endothelin levels were mediated through changes in the expression of genes controlling endothelin production, the levels of preproendothelin and endothelin-convert- ing enzyme (ECE) mRNA were assessed by relative RT-PCR and Northern blots. Finally, to further test the role of elevated endothelin levels in promoting lung water accumulation, hypoxic infected animals were treated with bosentan, a nonselective endothelin receptor antagonist. Gravimetric lung water, perivascular fluid cuffing, Evans blue-labeled albumin escape, and lung lavage fluid protein levels were then measured in animals given bosentan and compared with animals receiving vehicle alone.

METHODS

Animals. Experimental animals were pathogen-free weanling male Sprague-Dawley rats purchased from a commercial vendor (Harlan Sprague Dawley, Indianapolis, IN). Animals arrived in Denver at age 20–22 days and weighing 50–60 g

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each. Animals in the infected experimental groups were inoculated with virus 3–4 days after arrival and then housed in a separate facility from the noninfected animals. 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’s altitude (1600 m) at all times.

**Inoculation with Sendai virus.** Infection with Sendai virus was performed as previously described (1). Briefly, an isolate of Sendai virus was obtained from a commercial source (American Type Culture Collection, Manassas, VA). The lyophilized virus was resuspended and inoculated into rhesus monkey kidney cell culture tubes and incubated at 37°C in viral culture medium. After 72 h, the tubes were scraped and passed through one freeze-thaw cycle to cause cell lysis, and this crude extract was then centrifuged to remove cellular debris. The supernatant was then diluted to yield a concentration of $1 \times 10^7$ TCID$_{50}$/ml of virus, where TCID$_{50}$ is the 50% tissue culture infective dose, divided into aliquots, and stored frozen at $-70°C$ until used.

For inoculation with virus, animals were lightly sedated with methoxyflurane, and 50 μl of the virus stock solution ($5 \times 10^5$ TCID$_{50}$) were then instilled intranasally. Animals for uninfected experimental groups were treated in a similar manner and inoculated with a sham solution prepared in the same manner as the virus stock solution from rhesus monkey kidney cell cultures not inoculated with Sendai virus.

**Experimental design.** Animals were studied in four experimental groups: 1) normal control, 2) viral infection alone, 3) hypoxic exposure alone, and 4) viral infection combined with hypoxic exposure. Animals in the hypoxic test groups were weighed and then exposed to normobaric hypoxia (inspired $O_2$ fraction = 0.1) for 24 h. Exposure to hypoxia was timed so that all animals were killed on day 7 postinoculation (virus or sham) to compare animals at the same age and time point in the course of their Sendai infection.

Bosentan (generously provided by S. Roux, Hoffman-La Roche, Basel, Switzerland) was prepared as a microsuspension in 5% gum arabic (Sigma, St. Louis, MO) at a concentration of 25 mg/ml. Animals received a single oral dose of either gum arabic vehicle or bosentan (150 mg/kg) by gavage feeding 24 h before death. Hypoxia-exposed animals received bosentan or vehicle 30 min before the start of hypoxic exposure.

At the conclusion of the experimental protocol, animals were anesthetized with 80 mg/kg pentobarbital sodium given intraperitoneally. A midline thoracotomy was then performed, and the animal was exsanguinated via cardiac puncture. Blood was collected in heparinized syringes and placed on ice immediately. Plasma was collected after low-speed centrifugation (2,000 rpm for 10 min) and was stored at $-70°C$ for later testing. 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 extrapulmonary airways, snap-frozen in liquid nitrogen, and stored at $-70°C$ until processed for protein and mRNA assays. The right lungs from a separate series of animals treated in the same way were fixed in 10% buffered formalin by trancheal perfusion at 30 cmH$_2$O pressure for 24 h and then transferred to 70% ethanol before paraffin embedding and sectioning for histological studies. Calculations made were total lung weight-to-prehypoxia body weight ratios, expressed as milligrams of lung per gram of body weight, and wet-to-dry lung weight ratios.

**Endothelin assay.** Endothelin levels in lung homogenate and plasma were measured using a commercially available ELISA kit (Cayman Chemicals, Ann Arbor, MI). Frozen lung tissue collected as described above was homogenized at a ratio of 100 mg tissue/ml buffer in 50 mM Tris-HCl, pH 7.45, containing 0.1% Triton X, 1 μM leupeptin, 1 μM aprotinin, and 1 μM pepstatin. The homogenate was spun at 2,500 rpm for 10 min to remove crude debris, and the supernatant was saved as sample for the assay. Endothelin was extracted from the samples by mixing 10 μl of homogenate with 1 ml of ice-cold extraction solvent (acetone-2 N HCl-water in proportions 40:1:2). This mixture was spun in a microfuge at 12,500 rpm for 15 min, and the supernatant was decanted, dried in a vacuum centrifuge, and resuspended in the enzyme immunoassay buffer provided with the ELISA kit. The assay was then performed following the manufacturer’s instructions. In addition, samples spiked with a known quantity of endothelin were run with each assay as an internal control for the efficiency of the extraction. This procedure resulted in a 77 ± 3% recovery of endothelin from spiked samples.

The total protein content of the samples prior to extraction was measured by the Bradford method (Bio-Rad Laboratories, Hercules, CA) using bovine albumin standards, and results were expressed as picograms of endothelin per milligram of protein. Plasma samples were extracted by the addition of 200 μl of sample to 300 μl of extraction solvent (acetone-1 N HCl-water in proportions 40:1:5) and then assayed as described previously.

**Preproendothelin and ECE RT-PCR studies.** The expression of preproendothelin and ECE mRNAs was assessed by relative RT-PCR. Total lung RNA was extracted by the method of Chomczynski and Sacchi (4a). Briefly, frozen lung tissue was homogenized in TriReagent (Sigma), and the RNA was isolated following the manufacturer’s instructions. Isolated total lung RNA was then quantified by measurement in a spectrophotometer. Reverse transcription to cDNA was accomplished by priming 5 μg of total RNA per sample with oligo(dT), then using the Superscript II reverse transcriptase kit (GIBCO BRL, Gaithersburg, MD) to make cDNA, followed by digestion with RNase H to remove untranscribed RNA. PCR were performed on 1 μl of cDNA from each sample.

Primers were chosen for preproendothelin PCR based on the published coding sequence of the rat preproendothelin gene (GenBank accession number M64711). The sequences of the primers used were 5’-AGTGTGTCCTACTTGCCACTGGGCGG-3’ (bp 359–392) and 5’-TCCCAACCTCTGCTTGGAATTGTTTG-3’ (bp 974–951). The identity of the 606-bp PCR product generated with these primers was confirmed by restriction analysis and partial sequence analysis. As an internal control for each PCR, preproendothelin was coamplified with β-actin. Primers chosen for β-actin PCR were based on the published cDNA sequence of the mouse β-actin gene (GenBank accession number M12481). The sequences of the primers used were 5’-CATCGTAAAGAACCTCTTGCA-CCATG-3’ (bp 786–810) and 5’-CAAAGAAGGTGTTA-AAAAGAGC-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 product was confirmed by restriction analysis.

Duplex PCR for preproendothelin and β-actin was performed on 1 μl of cDNA per sample. Preproendothelin primers were used at 400 nM final concentration, and β-actin primers were used at 32 nM final concentration. Control reactions using increasing cycle numbers confirmed that the reaction for both products was in the exponential phase up to 23 cycles (data not shown), and all further studies were then done at 21 cycles. The PCR cycle used was 95°C for 45 s, 57°C for 45 s, and 72°C for 2 min. Contamination of the RNA samples with genomic DNA was excluded by the absence of
products from PCR performed using RNA without reverse transcription as well as by the absence of higher molecular weight bands indicating amplification of β-actin genomic DNA (data not shown).

Primers were chosen for ECE PCR based on the published sequence of the rat ECE gene (GenBank accession number D29683). The sequences of the primers used were 5'-TTCTGGATTGTGAGACCGTC-3' (bp 968–988) and 5'-CTATGTTCTTGTCCTCCG-3' (bp 1438–1417). The identity of the 471-bp PCR product for these primers was confirmed by restriction analysis. ECE was also coamplified with β-actin as an internal control using the same actin primers described above. Duplex PCR for ECE and β-actin was performed on 1 μl of cDNA per sample. ECE primers were used at 200 nM final concentration, and β-actin primers were used at 32 nM final concentration. Control reactions using increasing cycle numbers confirmed that the reaction for both products was in the exponential phase up to 23 cycles (data not shown), and all further studies were then done at 21 cycles. PCR cycle parameters and control reactions were as described previously for preproendothelin.

PCR products were loaded onto a 1.5% agarose-Tris-acetate-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 were expressed as the ratio of preproendothelin or ECE PCR product to β-actin PCR product for each sample. All experiments were repeated three times with similar results.

Preproendothelin and ECE Northern blotting studies. To confirm the results of our RT-PCR experiments, the expression of preproendothelin and ECE mRNA was also assessed by Northern blotting. Total lung RNA was isolated and quantified as described above. Northern blotting was performed using a commercial kit following the manufacturer’s instructions (Northern-Max Glyoxal, Ambion, Austin, TX). Briefly, 10 μg of RNA per sample were glyoxal treated, separated by electrophoresis in a 1% agarose gel, and transferred to positively charged nylon membranes (Hybond N+, Amersham) by downward alkaline transfer. Once transferred, the blots were UV cross-linked to fix the RNA to the membrane. Detection was accomplished using digoxigenin-labeled RNA probes. The blots were prehybridized for 30 min at 68°C in hybridization solution (UltraHyb, Ambion), and then a 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 using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Diagnostics, Indianapolis, IN) and CSPD chemiluminescent substrate.

The probes for preproendothelin and ECE were constructed using the primers listed above. The PCR products from these primers were ligated into the pCR1-TOPO vector (Invitrogen, Carlsbad, CA), and antisense RNA probes were produced by in vitro transcription using 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 ribosomal RNA probe transcribed from a commercially available template (Ambion). Results were expressed as the ratio of preproendothelin or ECE signal mRNA level to 18S RNA level for each sample. Northern blotting experiments were performed twice each with similar results.

Measurement of perivascular fluid cuff area. Measurements of perivascular fluid cuff areas were made on hema
toxylin and eosin-stained sections of lung tissue using a computer image analysis system (Nikon Optiphot 2 micro-
scope, Apple Macintosh Power Mac 7200 computer, and NIH Image, a public domain image analysis program developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Sections from three animals in each group were analyzed, and from each section, every vessel not contained in a bronchovascular sheath was measured. To minimize the confounding effects of distorted and collapsed vessels, the circularity of each vessel was also measured and those vessels with a circularity of less than 0.6 were excluded. Results were expressed as the percentage of vessels with a discernible fluid cuff, the absolute perivascular fluid cuff area, and the ratio of the pervascular fluid cuff area to the area of the vessel.

Measurement of intravascular protein leak. The leakage of protein from the vasculature was assessed by measuring the accumulation of Evans blue dye in the lung in an additional group of hypoxic infected animals treated with bosentan or vehicle alone as previously described. After exposure to 24 h of hypoxia, the animals were lightly anesthetized with methoxyflurane injected via a tail vein with 30 mg/kg of Evans blue dye and then returned to hypoxia for 10 min. After that time, the animals were anesthetized with 80 mg/kg of intraperitoneal pentobarbital sodium, the heart-lung block was excised, and the lungs were perfused free of blood with phosphate-buffered saline. The right lung was weighed and then incubated in formamide for 48 h at room temperature 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, and Evans blue dye extravasation was then expressed as nanograms of Evans blue per milligram of dry tissue.

Measurement of lung lavage fluid protein. Lung lavage fluid protein was measured on an additional group of hypoxic infected animals treated with bosentan or vehicle alone as previously described. At death, the lungs and main stem bronchi to the trachea were excised and weighed. The left main stem bronchus was then cannulated via the trachea, and lavaged with 2 ml of sterile phosphate-buffered saline in 1-ml aliquots. Returned lavage fluid was pooled and immediately placed on ice. Lavage fluid recovered from each animal was 1–1.5 ml. The fluid was centrifuged at low speed to remove cells and debris, and the total protein content of the supernatant was measured by the Bradford method (BioRad) using bovine albumin standards.

Confirmation of viral infection. Plasma obtained at death from all animals was tested for seroconversion to Sendai virus using an ELISA kit (ICN, Costa Mesa, CA). ELISA testing was performed at a serum dilution of 1:40, and results were interpreted by the difference in absorbance units at 405 nm between positive and negative test wells as follows: >150 = positive; >75 = weakly positive; ≤50 = negative.

Statistical analysis. Statistical analysis was performed using Statview 5.0 (SAS, Durham, NC) on an Apple Macintosh computer. Comparisons between the four experimental groups were made using a one-way ANOVA with Student-Newman-Keuls posttesting for multiple comparisons between groups. Comparisons between bosentan-treated and vehicle-treated animals were made using an unpaired Student’s t-test. Differences were considered statistically significant for P < 0.05. Results are expressed as means ± SE unless otherwise noted.
RESULTS

Effects of viral infection and hypoxia on lung water and lung endothelin content. Gravimetric lung water and lung homogenate endothelin content were measured for each of the four experimental groups. As in our previous study, animals exposed to virus plus hypoxia developed significantly higher lung weight-to-body weight ratios than the other four experimental groups (control 8.08 ± 0.07 mg/g, virus alone 7.94 ± 0.15, hypoxia alone 8.14 ± 0.06, virus plus hypoxia 8.9 ± 0.15; \( n = 9 \) per group; ANOVA for all 4 groups, \( P < 0.001 \); virus plus hypoxia vs. all others, \( P < 0.001 \)). Lung wet-to-dry weight ratios were also comparable to those in our previous report (control 4.86 ± 0.03, virus alone 4.88 ± 0.03, hypoxia alone 4.80 ± 0.03, virus plus hypoxia 4.95 ± 0.04; \( n = 5 \) per group; ANOVA for all 4 groups, \( P = 0.01 \)). Multiple comparison posttesting of the lung wet-to-dry weight ratio results revealed small but statistically significant differences between the virus plus hypoxia group and the hypoxia-alone group (\( P = 0.01 \)) and between the virus plus hypoxia group and the control group (\( P = 0.05 \)). Lung endothelin content was significantly elevated in the hypoxia-exposed groups, whereas animals exposed to viral infection alone had lung endothelin levels similar to normal control animals (Fig. 1; normal controls (control 0.33 ± 0.05, virus alone 0.34 ± 0.05, hypoxia alone 0.46 ± 0.1, hypoxia plus virus 0.5 ± 0.2; \( n = 4 \) per group; \( P = 0.34 \)). The combination of viral infection and hypoxia led to lung endothelin levels significantly higher than those for any of the other groups, including the group exposed to hypoxia alone. Lung endothelin content also demonstrated a statistically significant correlation with lung weight-to-body weight ratio for the four groups studied (Fig. 2). In contrast, plasma endothelin levels were similar among the four groups studied (control = 5.1 ± 0.3 pg/ml, virus alone = 4.6 ± 0.1, hypoxia alone = 4.8 ± 0.2, virus plus hypoxia = 5.0 ± 0.2; \( n = 4 \) per group; \( P = 0.34 \)).

Fig. 1. Lung endothelin content is increased in hypoxic and hypoxic infected animals. Values are shown as means ± SEM. Control, \( n = 9 \); virus alone, \( n = 9 \); hypoxia alone, \( n = 12 \); virus plus hypoxia, \( n = 12 \). *\( P < 0.03 \) vs. control and virus alone. †\( P < 0.001 \) vs. all other groups.

Effects of viral infection and hypoxia on lung preproendothelin and ECE mRNA expression. To assess whether the increase in lung endothelin peptide measured by ELISA in the hypoxic infected animals was associated with alterations in the expression of the genes controlling endothelin production, preproendothelin and ECE, lung mRNA levels for preproendothelin and ECE were assessed by relative RT-PCR. Preproendothelin mRNA levels were significantly elevated in the lungs of hypoxic infected animals compared with normal control animals (\( P = 0.02 \), Fig. 3). Lung ECE mRNA levels, in contrast, were significantly decreased in the hypoxic infected animals compared with normal controls (\( P = 0.03 \), Fig. 4).

To confirm the findings of these RT-PCR experiments, Northern blotting for preproendothelin and ECE mRNA was performed on the same samples, and the results were normalized to 18S ribosomal RNA on the same blot. With this technique, preproendothelin mRNA levels were significantly elevated in the lungs of hypoxic infected animals compared with normal controls (control 0.73 ± 0.22, hypoxic infected 2.06 ± 0.29; \( n = 4 \) each; \( P = 0.02 \)). Lung ECE mRNA levels as assessed by Northern blotting were decreased in the hypoxic infected animals compared with normal controls (control 0.33 ± 0.05, hypoxic infected 0.18 ± 0.02; \( n = 4 \) each; \( P = 0.03 \)).

Effect of endothelin receptor blockade on lung water. To further test the hypothesis that elevations in lung endothelin contributed to the increases in lung water found in the hypoxic infected animals, a second set of hypoxic infected animals was given bosentan, a nonselective endothelin receptor antagonist shortly before exposure to hypoxia. Bosentan-treated animals demonstrated significant decreases in lung weight-to-body weight ratio and in lung wet-to-dry weight ratio compared with vehicle-treated animals (Fig. 5). Lung endothelin content was similar between vehicle-treated and bosentan-treated animals (240 ± 31 pg endothelin/mg protein, \( n = 9 \), vs. 271 ± 40 pg endothelin/mg protein, \( n = 9 \)).
protein, \( n = 8, P = 0.72 \)). Bosentan-treated animals and vehicle-treated animals showed a similar decrease in body weight over the course of the hypoxic exposure (vehicle \(-11.8\%\) vs. bosentan \(-12.8\%, P = 0.37\)).

To confirm the measurements of gravimetric lung water, perivascular fluid cuffing was measured on paraffin-embedded, Formalin-fixed lung tissue from hypoxic infected animals given vehicle or bosentan. Histological examination of lung tissue from the hypoxic infected animals revealed amounts of perivascular fluid cuffing consistent with that found in our prior study (1). This fluid accumulation was most marked around vessels between 50 and 150 \( \mu \)m in diameter, but the findings were not significantly altered by including the small number of vessels counted above and below those sizes. Hypoxic infected animals subjected to endothelin receptor blockade with bosentan demonstrated significantly less perivascular fluid accumulation than hypoxic infected animals receiving vehicle alone (Table 1).

**Effect of endothelin receptor blockade on intravascular protein leak.** To assess whether endothelin receptor blockade reduced the leakage of protein from the vasculature in hypoxic infected animals, Evans blue dye extravasation into the lung was measured in hypoxic infected animals given vehicle or bosentan. Animals receiving bosentan showed significantly less lung Evans blue extravasation than animals receiving vehicle alone (14.2 \( \pm \) 1.6 vs. 8.8 \( \pm \) 1.4 ng Evans blue/mg dry lung; \( n = 4 \) per group; *\( P = 0.03 \)).

**Effects of endothelin receptor blockade on lung lavage fluid protein content.** To assess whether endothelin receptor blockade altered the accumulation of protein in the air spaces, lung lavage fluid protein content was measured on hypoxic infected animals given vehicle (\( n = 5 \)) or given bosentan (\( n = 5 \)). Animals receiving bosentan had significantly less total protein in their lung lavage fluid than animals receiving vehicle alone (Fig. 6).

**DISCUSSION**

The major findings of this study were that the combination of a recent viral respiratory infection and exposure to moderate environmental hypoxia not only increases lung water but also increases lung endothelin content in young rats. This elevation in lung endothelin content occurs in association with increases in lung preproendothelin mRNA levels and decreases in lung ECE mRNA levels and without a change in plasma endothelin levels. More importantly, endothelin receptor blockade in animals exposed to viral infection and hypoxia leads to significant decreases in gravimetric lung water, perivascular fluid cuffing, vascular permeability, and lung lavage fluid protein levels. These results suggest that increases in lung endothelin
content contribute to the lung water accumulation seen in this model.

We found that the endothelin content of the lung increases in hypoxic animals, a finding that is consistent with an earlier report in the literature (13). In the present study, exposure to hypoxia alone for 24 h was associated with a 40% increase in lung endothelin levels over control levels, and similar changes in lung endothelin peptide content have been previously reported in animals exposed to normobaric hypoxia alone for 48 h (13). Although a recent mild viral respiratory infection alone did not alter lung endothelin content in this study, viral infection did significantly augment the effect of hypoxia on lung endothelin levels. Interestingly, this change occurred despite the fact that the infected animals in this study were essentially asymptomatic at the time of exposure to hypoxia. The effects of viral respiratory infections on endothelins in the lung have not been studied previously in great detail. Consistent with our findings, however, increased levels of endothelin in the lung have been reported in mice infected with influenza virus for up to 4 days after infection, with those levels returning to baseline by day 8 of the infection (3). In addition, viral infections have been shown previously to alter the expression of endothelin receptors in airway smooth muscle in rats (2, 11), suggesting that viral infections may alter the endothelin effector system in the lung at several levels.

Although lung endothelin levels were markedly increased in the hypoxic and hypoxic infected animals, plasma endothelin levels were low and not different among the four groups studied. In contrast, most reports in the literature suggest that plasma endothelin levels increase with acute hypoxic exposure in both humans and experimental animals (6, 13). Prior work in human subjects has consistently shown small elevations in plasma endothelin levels with exposure to altitude (8, 14), although only one report has shown plasma endothelin levels to correlate with symptoms of HAPE (20). The reasons for the disparity between the present study and earlier reports are not immediately evident, although differences in the efficiency of the endothelin extraction methods used may play a role. Our findings suggest, nonetheless, that plasma measurements may not accurately reflect alterations in endothelin levels in lung tissue and that endothelins can have significant effects in the lung without detectable elevations in plasma levels.

The increases in endothelin peptide in the hypoxic infected animals were accompanied by increases in lung preproendothelin mRNA expression as assessed by relative RT-PCR. This finding is supported by earlier reports in the literature, although elevations in

Table 1. Endothelin receptor blockade decreases perivascular fluid cuffing in hypoxic infected animals

<table>
<thead>
<tr>
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<th>Hypoxia + Virus (n = 247)</th>
<th>Hypoxia + Virus + Bosentan (n = 243)</th>
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<td>77.8</td>
<td>45.2</td>
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<td>1,282 ± 190</td>
<td>0.0002</td>
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<td>Cuff area/vessel area</td>
<td>0.76 ± 0.07</td>
<td>0.38 ± 0.04</td>
<td>&lt;0.0001</td>
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Values are means ± SE; n, no. of vessels counted.

Fig. 5. Endothelin receptor blockade decreases lung water in hypoxic infected rats. A: lung weight-to-body weight ratio (LW/BW); n = 12/group. B: lung wet-to-dry weight ratio; n = 8/group.

Fig. 6. Endothelin receptor blockade decreases lung lavage fluid protein levels; n = 5/group.
lung preproendothelin mRNA levels have not been found previously with less than a 48-h exposure to hypoxia (6, 13). This finding again suggests, as with the increases in lung endothelin peptide, that the recent viral infection has exaggerated the response of the lung to hypoxia.

In light of the increase in lung endothelin peptide, the decrease in lung ECE mRNA is somewhat surprising. This finding might be explained if the change in ECE mRNA does not accurately reflect changes in ECE protein in the lung, which was not measured in this study. Other possibilities include that ECE is downregulated by the increased levels of endothelin in the lung, a pattern observed in cultured pulmonary artery endothelial cells (16), or that ECE is upregulated at specific sites within the lung but decreased in the lung as a whole, a pattern previously observed for angiotensin-converting enzyme expression (15). Alternatively, ECE expression may not significantly affect the level of endothelin in the lung in this setting. In support of this concept, previous studies of fetal lambs with pulmonary hypertension (10) and rats with heart failure (12) have shown increases in endothelin peptide and preproendothelin mRNA despite no change in ECE mRNA levels. The decreases in lung ECE mRNA levels in the hypoxic infected animals suggest then that the elevations observed in lung endothelin are not due to changes in ECE expression but rather are due to increased transcription of the preproendothelin gene, to decreased clearance of endothelin from the lung, or to both.

Two lines of evidence suggest that these elevations in lung endothelin content contribute directly to the lung water accumulation seen in this model. First, lung endothelin levels demonstrate a significant positive correlation with gravimetric lung water as reflected in the lung weight-to-body weight ratio. More importantly, blockade of the ET<sub>A</sub> and ET<sub>B</sub> receptors with bosentan prevented the increases in gravimetric lung water and markedly reduced the perivascular fluid cuffing, lavage fluid protein content, and lung Evans blue dye extravasation seen in the hypoxic infected animals. Taken together, these findings argue strongly that elevated levels of endothelin in the lungs of the hypoxic infected animals may promote early pulmonary edema formation. Alternatively, bosentan may have had a nonspecific effect on lung water, perhaps through promoting a change in overall body fluid balance. This possibility seems less likely, though, as the percent change in body weight during hypoxic exposure was not different between vehicle- and bosentan-treated animals.

Finally, our findings of decreased lung Evans blue dye extravasation and of less lung lavage fluid protein in the animals receiving bosentan suggest that endothelin receptor blockade may decrease the leakage of edema fluid out of the vasculature and into the air spaces. This study did not address, however, whether the effects of bosentan on lung water were mediated through altered pulmonary hemodynamics or through altered pulmonary vascular permeability. Altered pulmonary hemodynamics are likely in that bosentan has been shown to block the acute pulmonary artery pressure response to hypoxia in rats and to prevent pulmonary vascular remodeling in chronically hypoxic rats (4). Interestingly, however, bosentan also has been shown to reduce inflammation in models of allergic pneumonitis (7) and pulmonary fibrosis (18), suggesting that endothelin may have a role in moderating pulmonary inflammation and thus perhaps vascular permeability as well.

In summary, this study suggests that a recent mild respiratory infection can exaggerate the increases in lung endothelin content seen in young animals exposed to hypoxia, that those increases in lung endothelin can promote the development of pulmonary edema, and that endothelin receptor blockade can ameliorate the edema formation. Prior work has suggested that recent viral infections may be associated with HAPE in children and may augment lung water accumulation in young animals. Although the role of viral respiratory infections in human HAPE remains uncertain, evidence is accumulating that endothelin may be involved in the pathogenesis of this illness. Further study is needed to determine the mechanisms underlying the increases in pulmonary endothelin levels associated with the combination of viral infection and hypoxia as well as the clinical relevance of these findings.

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