Novel Peptide for Attenuation of Hyperoxia-induced Disruption of Lung Endothelial Barrier and Pulmonary Edema via Modulating Peroxynitrite Formation*

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Background: Hyperoxia increases NO and peroxynitrite in lung endothelium via increased interaction of eNOS with β-actin.

Results: P326TAT with sequence corresponding to actin binding region of eNOS residues 326–333 inhibited hyperoxia-induced disruption of endothelial barrier and apoptosis in cell culture and animal model.

Conclusion: P326TAT ameliorates barrier dysfunction of hyperoxic lung endothelium and pulmonary edema.

Significance: P326TAT can be a novel therapeutic agent to treat acute hyperoxic lung injury.

Pulmonary damages of oxygen toxicity include vascular leakage and pulmonary edema. We have previously reported that hyperoxia increases the formation of NO and peroxynitrite in lung endothelial cells via increased interaction of endothelial nitric oxide (eNOS) with β-actin. A peptide (P326TAT) with amino acid sequence corresponding to the actin binding region of eNOS residues 326–333 has been shown to reduce the hyperoxia-induced formation of NO and peroxynitrite in lung endothelial cells. In the present study, we found that exposure of pulmonary artery endothelial cells to hyperoxia (95% oxygen and 5% CO2) for 48 h resulted in disruption of monolayer barrier integrity in two phases, and apoptosis occurred in the second phase. NOS inhibitor N\(^{\text{G}}\)-nitro-\(L\)-arginine methyl ester attenuated the endothelial barrier disruption in both phases. Peroxynitrite scavenger uric acid did not affect the first phase but ameliorated the second phase of endothelial barrier disruption and apoptosis. P326TAT inhibited hyperoxia-induced disruption of monolayer barrier integrity in both phases and apoptosis in the second phase. More importantly, injection of P326TAT attenuated vascular leakage, pulmonary edema, and endothelial apoptosis in the lungs of mice exposed to hyperoxia. P326TAT also significantly reduced the increase in eNOS-β-actin association and protein tyrosine nitration. Together, these results indicate that peptide P326TAT ameliorates barrier dysfunction of hyperoxic lung endothelial monolayer and attenuates eNOS-β-actin association, peroxynitrite formation, endothelial apoptosis, and pulmonary edema in lungs of hyperoxic mice. P326TAT can be a novel therapeutic agent to treat or prevent acute lung injury in oxygen toxicity.

Oxygen toxicity is the most severe side effect of oxygen therapy in neonatal and adults. Prolonged exposure to higher concentrations of oxygen induces diffuse pulmonary injuries, vascular leakage, excessive inflammation, and pulmonary edema (1, 2). Effective therapy is lacking because the molecular mechanism of hyperoxia-induced vascular leakage is not completely understood. The hyperoxia-induced damages to lung cells have been attributed to the generation of reactive oxygen species and subsequent formation of more potent oxidants such as peroxynitrite (3–5). Reactive oxygen species generated during hyperoxia have been shown to increase lung vascular leakage due to endothelial injuries in oxygen toxicity (6–8). Peroxynitrite-induced protein tyrosine nitration is associated with increased apoptosis in microvascular endothelium (9, 10). Blocking protein tyrosine nitration prevents vaso-obliteration and neovascularization in retinopathy (10) and apoptosis of hepatic stellate cells (11).

We have previously reported that hyperoxia increases the formation of NO and peroxynitrite in lung endothelial cells via increased interaction of eNOS with β-actin (12). The actin binding site on eNOS protein has been identified at amino acid residues 326–333. Mutation of hydrophobic residues leucine 326, leucine 328, tryptophan 330, and leucine 333 resulted in dissociation of eNOS from β-actin, indicating that these residues are critical for actin binding (13). A peptide with amino acid sequence corresponding to the actin binding region of eNOS residues 326–333 has been shown to reduce the hyperoxia-induced formation of NO and peroxynitrite in lung endothelial cells (12). In the present study, a peptide (P326TAT) with amino acid sequence corresponding to the actin binding region of eNOS residues 326–333 linked to a 10-amino acid transduction domain of HIV-tat (RKKKRQRRRA) was synthesized, and its effects on hyperoxia-induced disruption of lung endothelial barrier were studied using cultured endothelial cells and animal model. The results indicate...
that peptide P326TAT ameliorates endothelial barrier dysfunction of hyperoxic lung endothelial monolayer and attenuates peroxynitrite formation, endothelial apoptosis, and pulmonary edema in hyperoxia-induced lung injury in mice of oxygen toxicity.

**EXPERIMENTAL PROCEDURES**

**Reagents and Materials**—Mouse anti-eNOS antibody was obtained from BD Biosciences. Antibody against endothelial marker von Willebrand factor was from NOVUS Biologicals (Littleton, CO). Nitrotyrosine antibody was from Millipore (Billerica, MA). Apoptosis-inducing factor (AIF) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). i-NAMe was from Calbiochem. Anti-β-actin monoclonal antibody, uric acid, and other reagents were purchased from Sigma unless specified otherwise.

**Cell Culture**—Primary bovine pulmonary artery endothelial cells (PAECs) were obtained from Cell Applications (San Diego, CA). Cells were cultured in F12K medium supplemented with 10% FBS and antibiotics. Passages from 2 to 10 were used for experiments.

**Oxygen Exposure**—Confluent PAECs were exposed to normoxia (room air, 5% CO2) or to hyperoxia (95% oxygen, 5% CO2) in a sealed chamber for 1–48 h in F12K medium containing 4% FBS.

**Endothelial Monolayer Barrier Assay**—An ECIS-Zeta electric cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) was used to determine transendothelial electrical resistance (TEER) value as a measurement of endothelial barrier integrity of PAEC monolayer. Confluent endothelial cells were seeded on gold-coated electrodes arrays and equilibrated in F12K medium containing 4% FBS overnight. After resistances were relatively constant (1000–1200 ohms), treatments (uric acid, i-NAMe, or peptides) were added directly to the wells and arrays were allowed to equilibrate for another 3 h. Then, cells were exposed to hyperoxia (95% oxygen, 5% CO2) for 48 h. The impedance was recorded for the duration of the experiments. Data were normalized to the mean resistance before hyperoxia exposure.

**Apoptosis Assay**—TUNEL staining and caspase-3 assay were used to detect apoptosis. In TUNEL assay, PAECs were plated on Lab-Tek 8-well chamber slides (Thermo Fisher). After exposure to hyperoxia, apoptosis was detected using ApopTag Plus fluorescent in situ apoptosis detection kit (Millipore, Temecula, CA) according to the manufacturer’s protocol. TUNEL staining was also performed on paraffin-embedded lung sections using the same kit. The slides were subjected to TUNEL staining, which was followed by staining with antibody against von Willebrand factor conjugated with Alexa Fluor 594. Nuclei were stained with DAPI. Slides were evaluated by Zeiss LSM 510 laser scanning confocal microscope. Caspase-3 activity was measured by microplate reader at 405 nm.

**Synthesis of Actin Binding Sequence Peptides**—Peptide P326TAT with amino acid sequence corresponding to the actin binding region of eNOS residues 326–333 linked to a 10-amino acid transduction domain of HIV-tat (RKKRRQRRRA) was synthesized by GenScript Corp. (Piscataway, NJ). Fusion of this 10-amino acid TAT tag is to facilitate delivery of this peptide into cells and tissue (13, 14). A modified version of actin binding sequence peptide 326 with hydrophobic leucine and tryptophan substituted for neutrally charged alanine was used as a control peptide (PlwTAT). The amino acid sequences of the peptides are RKKRRQRRRALGLRWYAL for P326TAT and RKKRRQRRRAAGARAYAA for PlwTAT. The peptides were synthesized by GenScript.

**Transfection of Endothelial Cells with the Peptides**—PAECs were incubated with P326TAT or PlwTAT peptides at 20 μm final concentration in F12K medium supplemented with 4% FBS. After 1 h of incubation, cells were exposed to hyperoxia or normoxia for 48 h before being used for TUNEL assay. In some experiments, endothelial monolayer permeability was monitored for up to 48 h.

**Isolation of Nuclear Fraction**—After hyperoxic exposure, PAECs were collected and the nuclear fraction was isolated using a NE-PER nuclear protein extraction kit (Pierce Biotechnology).

**Exposure of Mice to Hyperoxia**—Male C57BL/6 mice and breeding pairs of eNOS knock-out mice (strain B6.129P2-Nos3 tm1Unc/J, stock number 002684) between 8 and 10 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME). All experiments were performed in accordance with the guiding principles of the National Institutes of Health Guide for the Case and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the Georgia Regents University. For hyperoxic exposure, mice were kept in a clear plastic polypropylene chamber (30 × 20 × 20 inches) with free access to food and water. The oxygen concentration in the chamber was maintained using a ProOx oxygen controller (Biospherix, Lacona, NY) at 80% for 5 days. The concentration of CO2 in the chamber was lower than 0.3%. Mice were given either P326TAT peptide or control peptide PlwTAT intraperitoneally, daily, 10 mg/kg.

**Mouse Lung Experiments**—Mice were anesthetized (pentobarbital, 90 mg/kg, intraperitoneally), and the trachea was intubated. The mice were then euthanized by using thoracotomy. The blood in pulmonary circulation was rinsed by infusing PBS through pulmonary artery. Then, lungs were removed, fixed in 4% paraformaldehyde, and embedded in paraffin. Alternatively, lungs were removed and snap-frozen in liquid nitrogen. The analysis of nitrotyrosine level and eNOS/actin co-immunoprecipitation was performed from frozen samples. Paraffin-embedded sections were used for H&E staining and for TUNEL assay and immunostaining against von Willebrand factor. Fluorescence images were acquired using a confocal laser scanning microscope LSM 510 as described above. The fluorescence intensity was measured using the software ImageJ.

**Vascular Leakage Assessment**—After hyperoxic exposure, mice were injected with Evans blue dye (EBD) (20 mg/kg) via the tail vein (15). After 30 min, the mice were then anesthetized using pentobarbital and euthanized by using thoracotomy. The blood in pulmonary circulation was rinsed by infusing PBS.
through pulmonary artery. The lungs were excised en bloc and weighed. The lungs were then dried for 48 h in an oven at 65 °C and were weighed again. Lung tissue was manually homogenized using a bead beater (BioSpec Products, Bartlesville, OK) and incubated with double the volume of formamide for 18 h at 60 °C. After centrifugation at 5,000 × g for 30 min, the supernatant was collected. The optical density of the supernatant was determined by a spectrophotometer at 620 nm. Levels of EBD in samples were quantitated using a standard curve prepared with known amounts of EBD and are expressed as ng/mg of protein. Wet to dry lung weight ratio was calculated as a measure of lung tissue water content expressed as wet to dry ratio adjusted to total body weight.

**Statistical Analysis—**In each experiment, control and experimental cells were matched for cell line, age, seeding density, number of passages, and number of days after confluence to avoid variation in tissue culture factors that can influence measurements. Results are shown as means ± S.E. for n experiments. One-way analysis of variance and post t test analyses were used to determine the significance of differences between the means of different groups. p < 0.05 was considered statistically significant.

**RESULTS**

**Exposure of PAECs to Hyperoxia Disrupts Endothelial Monolayer Barrier Function—**PAECs were exposed to hyperoxia (95% O₂ and 5% CO₂) or normoxia (air and 5% CO₂), and TEER was measured using an ECIS-Zeta electric cell-substrate impedance sensing system. Exposure of PAECs to 95% oxygen for 48 h resulted in decrease in TEER in two phases (Fig. 1A). In the first phase, the TEER started to decrease at 1 h and reached the lowest at 3 h after starting exposure. In the second phase, the TEER began to decrease at 24 h after hyperoxic exposure and exhibited an irreversible decrease after exposure for 48 h (Fig. 1A).

**NOS Inhibitor L-NAME Prevents Hyperoxia-induced Disruption of Lung Endothelial Barrier—**PAECs were exposed to hyperoxia or normoxia for 48 h in the presence and absence of NOS inhibitor L-NAME (3 mM). We found that L-NAME prevented decrease in TEER in both phases in hyperoxic PAECs (Fig. 1B), suggesting that the two-phase barrier disruption of hyperoxic lung endothelial monolayer is caused by increased NO production.

**Peroxynitrite Scavenger Uric Acid Prevents Hyperoxia-induced Disruption of Lung Endothelial Barrier in the Second Phase and Apoptosis—**Exposure of lung endothelial cells to high concentration of oxygen leads to peroxynitrite formation (12). To investigate whether hyperoxia-induced disruption of lung endothelial barrier is caused by peroxynitrite, PAECs were exposed to hyperoxia or normoxia for 48 h in the presence and absence of peroxynitrite scavenger uric acid (3 mM). We found that uric acid did not affect hyperoxia-induced decrease in TEER in the first phase (Fig. 2A). However, uric acid prevented the decrease in TEER in hyperoxic PAECs in the second phase (Fig. 2A). These results suggest that peroxynitrite contributes to the barrier disruption of lung endothelial cells in the second phase but not in the first phase.

We studied whether hyperoxia-induced disruption of lung endothelial barrier function involves apoptosis of endothelial cells. Apoptosis was evaluated using TUNEL assay. Exposure of PAECs to hyperoxia for 5 h did not cause apoptosis of lung endothelial cells (data not shown). However, exposure to hyperoxia for 48 h resulted in a significant increase in the number of TUNEL-positive cells (Fig. 2B), suggesting that long-term exposure to higher concentration causes apoptosis of lung endothelial cells, which may be related to endothelial barrier dysfunction in the second phase. Furthermore, treatment of PAECs with uric acid inhibited hyperoxia-induced increase in the number of TUNEL-positive cells (Fig. 2B), indicating that hyperoxia-induced apoptosis of lung endothelial cells is attributable to peroxynitrite.

**Peptide P326TAT Attenuates Hyperoxia-induced Disruption of Lung Endothelial Barrier—**We have previously shown that specific disruption of eNOS-β-actin interaction using peptide P326TAT prevents hyperoxia-induced NO and peroxynitrite production and tyrosine nitration in intact endothelial cells (12). NO and peroxynitrite have been shown to affect endothelial barrier function (19, 20). To study whether modulation of eNOS-actin interaction by using peptide P326TAT has an effect on hyperoxia-induced barrier dysfunction of lung endo-
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FIGURE 2. Uric acid prevents hyperoxia-induced disruption of lung endothelial barrier in the second phase and apoptosis. PAECs were treated with and without uric acid (3 mM) and exposed to hyperoxia for 48 h, during which TEER was continuously monitored. After exposure, apoptotic cells were detected using TUNEL assay as described under “Experimental Procedures.” A, changes in TEER of endothelial monolayer. *, alterations in the numbers of TUNEL-positive cells. Results are expressed as means ± S.E.; n = 4, * p < 0.05 versus normoxia; #, p < 0.05 versus UA + normoxia; **, p < 0.05 versus hyperoxia. UA = uric acid.

FIGURE 3. Peptide P326TAT attenuates hyperoxia-induced disruption of endothelial monolayer barrier integrity. PAECs were treated with peptide P326TAT (20 μM) or control peptide PlwTAT (20 μM) and exposed to hyperoxia for 48 h. TEER was continuously monitored as described under “Experimental Procedures.” A, changes in TEER of endothelial monolayer under hyperoxia with control peptide PlwTAT. B, changes in TEER of endothelial monolayer under hyperoxia with P326TAT. Results are expressed as means ± S.E.; n = 4, * p < 0.05 versus normoxia; #, p < 0.05 versus P326TAT + normoxia or PlwTAT + normoxia; **, p < 0.05 versus hyperoxia.

Endothelial cells, PAECs were pretreated with peptide P326TAT (20 μM) or control peptide PlwTAT (20 μM) and then exposed to hyperoxia. As shown in Fig. 3B, treatment of PAECs with P326TAT reduced hyperoxia-induced decrease in TEER in both phases. The control peptide PlwTAT did not influence the effect of hyperoxia on TEER (Fig. 3A). These data indicate that peptide P326TAT attenuates hyperoxia-induced disruption of lung endothelial barrier.

Peptide P326TAT Attenuates Hyperoxia-induced Apoptosis of Lung Endothelial Cells—PAECs were exposed to hyperoxia or normoxia in the presence of P326TAT (20 μM) or control peptide PlwTAT (20 μM) for 48 h, and then apoptosis was evaluated using TUNEL assay. Treatment of PAECs with P326TAT inhibited hyperoxia-induced increase in the number of TUNEL-positive cells (Fig. 4), indicating that P326TAT attenuates hyperoxia-induced apoptosis of lung endothelial cells.

Effect of Peptides P326TAT and PlwTAT on Hyperoxia-induced Acute Lung Injury and Pulmonary Edema in Wild-type and eNOS Knock-out Mice—Wild-type and eNOS knock-out male mice were treated daily with P326TAT (intraperitoneally, 10 mg/kg) or PlwTAT (intraperitoneally, 10 mg/kg) and exposed to 80% oxygen or room air for 5 days. Lung morphology, lung wet/dry ratio, lung microvascular leakage, and lung endothelial apoptosis were assessed. The lungs from wild-type hyperoxic mice receiving control peptide PlwTAT exhibited inflammatory infiltrations, edema, and alveolar wall thickening (Fig. 5A). Injection of P326TAT ameliorated the morphological alterations in the lungs of wild-type hyperoxic mice (Fig. 5A). Moreover, lung wet/dry ratio and EBD leakage from lung vessels are much higher in wild-type hyperoxic mice receiving control peptide PlwTAT (Fig. 5, B and C). P326TAT significantly reduced the increases in lung wet/dry ratio and EBD leakage from lung vessels in wild-type mice exposed to hyperoxia (Fig. 5, B and C). These results indicate that P326TAT ameliorates hyperoxia-induced acute lung injury and pulmonary edema in mouse lungs.

The lungs from eNOS knock-out hyperoxic mice receiving control peptide PlwTAT did not exhibit obvious inflammatory infiltrations, edema, and alveolar wall thickening (Fig. 5A). There was no significant difference in lung morphology between eNOS knock-out hyperoxic mice receiving control peptide PlwTAT and those receiving P326TAT (Fig. 5A). Further, lung wet/dry ratio and EBD leakage from lung vessels are lower in eNOS knock-out hyperoxic mice receiving control peptide PlwTAT (Fig. 5, B and C). There was no significant difference in lung wet/dry ratio and EBD leakage from lung vessels between eNOS knock-out hyperoxic mice receiving control peptide PlwTAT and those receiving P326TAT (Fig. 5, B and C). Taken together, these results indicate that eNOS plays an important role in hyperoxia-induced acute lung injury and pulmonary edema and that the attenuating effect of P326TAT on hyperoxia-induced acute lung injury and pulmonary edema is through eNOS.

Effect of Peptides P326TAT and PlwTAT on Hyperoxia-induced Endothelial Apoptosis in Mouse Lungs of Wild-type and eNOS Knock-out Mice—We found that hyperoxic exposure resulted in higher numbers of TUNEL-positive endothelial cells in lungs of wild-type mice treated with control peptide PlwTAT
Peptide P326TAT attenuates hyperoxia-induced apoptosis of lung endothelial cells. PAECs were exposed to hyperoxia or normoxia in the presence of P326TAT (20 μM) or control peptide PlwTAT (20 μM) for 48 h, and then apoptosis was evaluated using TUNEL assay. A, representative images of TUNEL staining. WO peptide, without peptide. B, bar graph depicting the changes in the numbers of TUNEL-positive cells. Results are expressed as means ± S.E.; n = 4. *, p < 0.05 versus normoxia; #, p < 0.05 versus PlwTAT + hyperoxia or hyperoxia only.
and that P326TAT reduced the numbers of TUNEL-positive endothelial cells in lungs of wild-type hyperoxic mice (Fig. 6), suggesting that P326TAT inhibits apoptosis of endothelial cells in the hyperoxic mouse lungs. Further, there was no significant difference in the numbers of TUNEL-positive lung endothelial cells between eNOS knock-out hyperoxic mice receiving control peptide PlwTAT and those receiving P326TAT (Fig. 6), further supporting that eNOS plays an important role in hyperoxia-induced acute lung injury and that the attenuating effect of P326TAT on hyperoxia-induced acute lung injury is through eNOS.

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**FIGURE 5.** Effect of peptides P326TAT and PlwTAT on hyperoxia-induced acute lung injury and pulmonary edema in wild-type and eNOS knock-out mice. Wild-type (WT) and eNOS knock-out (KO) male mice were treated daily with P326TAT (intraperitoneally, 10 mg/kg) or PlwTAT (intraperitoneally, 10 mg/kg) and exposed to 80% oxygen or room air for 5 days. Lung morphology, lung wet/dry ratio, and lung microvascular leakage were assessed described under “Experimental Procedures.” A, representative images of lungs in H&E staining. B, bar graph depicting changes in lung wet/dry ratio. C, bar graph depicting changes in EBD leakage. Results are expressed as means ± S.E.; n = 8. *, p < 0.05 versus normoxia; #, p < 0.05 versus hyperoxia in P326TAT group.

**FIGURE 6.** Effect of peptides P326TAT and PlwTAT on hyperoxia-induced endothelial apoptosis in mouse lungs of wild-type and eNOS knock-out mice. Wild-type (WT) and eNOS knock-out (KO) male mice were treated daily with P326TAT (intraperitoneally, 10 mg/kg) or PlwTAT (intraperitoneally, 10 mg/kg) and exposed to 80% oxygen or room air for 5 days. Lung sections were stained for TUNEL and von Willebrand factor as described under “Experimental Procedures.” A, representative images of TUNEL (green) and von Willebrand factor (red). B, bar graph depicting changes in the fluorescence intensity of the green area close to the red area. Results are expressed as means ± S.E.; n = 8. *, p < 0.05 versus normoxia.

Peptide P326TAT Reduces Hyperoxia-induced Increases in eNOS-β-Actin Interaction and Protein Tyrosine Nitration in Mouse Lungs—In a previous study, we demonstrated that hyperoxia increases eNOS-β-actin association and protein tyrosine nitration in mouse lungs (12). Here, we analyzed whether the protective effect of P326TAT on hyperoxia-induced acute lung injury and pulmonary edema involves its effect on eNOS-β-actin interaction and protein tyrosine nitration in mouse lungs. Consistent with our previous observation, exposure of mice receiving control peptide PlwTAT to 80% oxygen for 5 days resulted in an increase in eNOS-β-actin interaction in lung tissue as detected by immunoprecipitation (Fig. 7). Meanwhile, tyrosine nitration of several proteins in lung homogenates of mice treated with control peptide was elevated (Fig. 8). Treatment with P326TAT attenuated increases in eNOS-β-actin association and protein tyrosine nitration level in the lungs of hyperoxia-exposed mice (Figs. 7 and 8). Together, these data demonstrate that reduction of eNOS-β-
Peptide P326TAT Prevents Caspase-dependent and -independent Apoptosis of Lung Endothelial Cells—Treatment of PAECs with P326TAT but not with PlwTAT prevented hyperoxia-induced increase in caspase-3 activity (Fig. 9A). We also found that hyperoxia induced increase in protein contents of AIF in the nuclear fraction of lung endothelial cells (Fig. 9B), suggesting that hyperoxia causes caspase-independent apoptosis. Treatment of PAECs with P326TAT prevented increase in intranuclear AIF protein levels in hyperoxic PAECs (Fig. 9B). These data demonstrate that peptide P326TAT prevents caspase-dependent and -independent apoptosis of lung endothelial cells.

DISCUSSION

The goal of this study was to determine the effect of peptide P326TAT on lung endothelial barrier disruption in oxygen toxicity. To this end, we utilized two models: a cell model of PAECs exposed to 95% oxygen and an animal model of mice exposed to 80% hyperoxia for 5 days. We found that hyperoxia induced a two-phase disruption of lung endothelial barrier integrity and that peptide P326TAT ameliorates both phases of endothelial barrier dysfunction and endothelial apoptosis. More importantly, P326TAT attenuates peroxynitrite formation, endothelial apoptosis, and pulmonary edema in hyperoxia-induced lung injury in mice of oxygen toxicity. These results indicate that P326TAT can be a novel therapeutic agent to treat or prevent hyperoxia-induced acute lung injury.

Several studies have confirmed that exposure of lung endothelial cells to high concentration of oxygen causes disruption of endothelial barrier (8, 17, 18). In those studies, the endothelial barrier function was assessed by measuring albumin leakage through endothelial monolayer (8, 17). In the present study, we continuously monitored the endothelial barrier function by detecting changes in TEER of PAEC monolayer in real time. We found that prolonged exposure of PAECs to hyperoxia resulted in a two-phase barrier disruption of endothelial monolayer. In the first phase, the endothelial barrier dysfunction starts at 1 h and reached lowest at 3 h after starting exposure. The second phase barrier disruption of endothelial monolayer is irreversible and occurs after hyperoxic exposure for 24 h. Meanwhile, apoptosis is detected in this phase, suggesting that cell death contributes to the second phase disruption of endothelial barrier integrity.

NO has been shown to increase endothelial permeability (19, 20), and eNOS activity is increased in PAECs exposed to hyperoxia for as early as 1 h (12). Interestingly, we found that NOS inhibitor l-NAME attenuates both phases of endothelial barrier disruption and that eNOS knock-out inhibits hyperoxia-induced acute lung injury and pulmonary edema in mouse lungs, suggesting that increased NO release contributes to both phases of disruption of hyperoxic lung endothelial monolayer and hyperoxic lung injury. Exposure of lung endothelial cells to high concentration of oxygen leads to accumulation of a large amount of reactive oxygen species (5). The superoxide radicals react with NO generated by eNOS and form more potent oxidizing reactive nitrogen species such as peroxynitrite. eNOS plays an important role in peroxynitrite formation and protein tyrosine nitration in endothelial cells during hyperoxia (4, 9, 21). l-Arginine, a substrate for NOS, enhances injury in the isolated rabbit lung during hyperoxia (22). Inhibition of eNOS using l-NAME or knock-out of eNOS reduces peroxynitrite-
mediated cytotoxicity in hyperoxic retinal damage (9, 23). In the present study, we found that peroxynitrite scavenger uric acid prevents the second phase of endothelial barrier disruption and PAEC apoptosis. Taken together, increase in NO release contributes to the first phase disruption of hyperoxic lung endothelial monolayer, and the second phase disruption of hyperoxic lung endothelial monolayer and apoptosis are attributable to the increase in peroxynitrite formation.

We have previously reported that hyperoxia increases the formation of NO and peroxynitrite in lung endothelial cells via increased interaction of eNOS with β-actin in oxygen toxicity (12). The actin binding site on eNOS protein has been identified at amino acid residues 326–333 (13). Mutation of hydrophobic residues leucine 326, leucine 328, tryptophan 330, and leucine 333 resulted in dissociation of eNOS from β-actin, indicating that these residues are critical for actin binding (13). P326TAT, a peptide with amino acid sequence corresponding to the actin binding region of eNOS residues 326–333, has been shown to readily enter endothelial cells (13). Incubation of PAECs with P326TAT blocks eNOS-β-actin association in hyperoxic PAECs and subsequently reduces the hyperoxia-induced formation of NO and peroxynitrite (12). To study whether modulation of eNOS-β-actin interaction by P326TAT has an effect on hyperoxia-induced barrier dysfunction of lung endothelial cells, PAECs were pretreated with P326TAT or control peptide PlwTAT and then exposed to hyperoxia. We found that P326TAT attenuates hyperoxia-induced disruption of lung endothelial barrier integrity in both phases. This effect is not due to the nonspecific action of the peptide because control peptide PlwTAT does not influence the effect of hyperoxia on endothelial barrier. Moreover, P326TAT inhibits hyperoxia-induced endothelial apoptosis in the second phase of barrier disruption. Therefore, inhibition of the hyperoxia-induced formation of NO and peroxynitrite by P326TAT prevents barrier disruption and apoptosis of lung endothelial cells exposed to hyperoxia.

Prolonged exposure to increased concentrations of oxygen induces diffuse pulmonary injuries, excessive inflammation, and lung fibrosis (2, 24). Lung vascular endothelial alterations represent the most striking pathophysiological changes in hyperoxia-induced lung injuries (25). In the animal experiment, we found that P326TAT ameliorates endothelial apoptosis, edema, alveolar wall thickening, and inflammatory infiltrations in the lungs of mice exposed to hyperoxia. Our data provide the first evidence supporting that P326TAT is a potential novel agent to treat or prevent lung injury in oxygen toxicity.

To study the mechanism that is responsible for the attenuating effect of P326TAT on hyperoxia-induced acute lung injury, we studied the effect of peptide P326TAT and control peptide PlwTAT on hyperoxia-induced acute lung injury and pulmonary edema in wild-type and eNOS knock-out mice. We found that there was no significant difference in lung morphology, lung wet/dry ratio, EBD leakage from lung vessels, and the numbers of TUNEL-positive lung endothelial cells between eNOS knock-out hyperoxic mice receiving PlwTAT and those receiving P326TAT. Thus, the attenuating effect of P326TAT on hyperoxia-induced acute lung injury and pulmonary edema is through eNOS. We have previously reported that mouse lungs exposed to hyperoxia exhibit increases in eNOS-β-actin association and protein tyrosine nitration (12). Our current observations show that P326TAT reduces hyperoxia-induced increases in eNOS-β-actin association and protein tyrosine nitration in mouse lungs. Therefore, peroxynitrite formation caused by increased eNOS activity because of eNOS-β-actin association plays an important role in lung vascular endothelial damage in hyperoxic lung injuries.

Apoptosis occurs in a caspase-dependent and -independent fashion. Chromatin condensation and DNA degradation triggered by AIF represent the most important caspase-independent apoptotic pathway (26). We found that caspase-3 activity is increased and AIF protein level in nuclei is significantly higher in PAECs exposed to hyperoxia. Furthermore, P326TAT prevents increases in caspase-3 activity and nuclear AIF protein level hyperoxic PAECs, suggesting that inhibition of peroxynitrite formation by P326TAT prevents both caspase-dependent and caspase-independent apoptosis. These results provide further evidence that inhibition of peroxynitrite formation by P326TAT prevents hyperoxia-induced lung endothelial injury in oxygen toxicity.

In summary, we have shown that inhibition of NO and peroxynitrite formation by peptide P326TAT prevents barrier disruption and apoptosis of lung endothelial cells exposed to hyperoxia. P326TAT attenuates nitrosative stress, endothelial...
apoptosis, and pulmonary edema in hyperoxic lungs. P326TAT can be a novel therapeutic agent to treat or prevent acute lung injury in oxygen toxicity.

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